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PEOPLE AND EVENTS

L. A. Black Recipient of Citation

L. A. BLACK of the U. S. Public Health Service has been given the Citation Award of the International Association of Milk and Food Sanitarians. The presentation was made by H. B. ROBINSON, senior past-president of the Association and chairman of the Committee on Recognition and Awards, at the 47th Annual Meeting of the Association in Chicago on October 27, 1960.

The Citation Award is given annually to an individual who has given outstanding service to the Association, and who has advanced the cause of sanitation and the sanitarian. The award was given to Dr. Black, who is Chief of Milk Sanitation, Milk and Food Research, at the Robert A. Taft Sanitary Engineering Center, Cincinnat., for his work in the development and unification of laboratory methods and standards in milk and food sanitation.

Dr. L. A. Black presented with Citation by Harold Robinson.

The Citation reads: "For outstanding service to the Association in providing leadership in the development and unification of milk and food sanitation laboratory methods and standards through frequent contribution of significant articles to the Journal of Milk and Food Technology; through service as an Associate Editor of the Journal; through service as a member and chairman of the Committee on Applied Laboratory Methods; and through service as Association representative to groups concerned with the standardization of laboratory equipment."

Dr. Black, a native of Macon, Illinois, is a graduate of the University of Illinois, receiving his bachelor's degree in 1924, his M.S. degree in 1925, and his Ph.D. degree in 1928. After serving as a bacteriologist with the Illinois State Health Department from 1926 to 1927, he became an instructor and assistant professor of Bacteriology at the State College of Washington, serving also as associate dairy bacteriologist in charge at the Washington Agricultural Experiment Station from 1928 to 1930.

From 1930 to 1941 Dr. Black was associate professor and professor of Bacteriology at the University of Maryland. In the latter year he joined the Public Health Service at the Sanitary Engineering Center.

Dr. Black is also a member of the Society of American Bacteriologists, the American Dairy Science Association, and is a fellow of the American Public Health Association. He is now chairman of American Public Health Association Subcommittee on Standard Methods for the Examination of Dairy Products, chairman of the Laboratory Committee of the National Conference for Interstate Milk Shipments, and is a member of the Public Health Committee of the American Dairy Science Association.

He is recognized as an authority in milk and food sanitation bacteriology. Research conducted by him includes collaborative studies in the development and standardization of laboratory methods for the examination of milk, studies leading to the development of methods for the testing of bactericides, and extensive studies for evaluating the performance of laboratories. Dr. Black's contributions to the scientific literature include more than 80 articles.

W. D. McMillan Passes Away August 9, 1960

W. D. MCMILLAN, Ithaca, New York, was born in Schenectady County, New York, October 10, 1899. He was graduated from Cornell University in 1924, with a major in animal husbandry and agricultural economics, and received his Master's degree in animal nutrition.

Since McMillan joined G.L.F. in 1924 he has worked on developing G.L.F. feed formulas, working on recommendations from state agricultural college nutrition authorities. He was manager of Agricultural Advertising and Research Service, Inc., which handled G.L.F. advertising for several years. He is a past-president of the Feed Industry Council, now the Feed Survey Committee, sponsored by the American Feed Manufacturers Association, Chicago, Illinois, and has served on the Feed Technical Advisory Committee and the Na-

W. D. McMillan

tional Pet Food Institute, of the A.F.M.A. He also served on the Feed Survey Committee set up by the A.F.M.A. during World War II to allocate use of feedstuffs. He served as president of this Committee for 1 yr. He is a member of the Poultry Science Association, the Society of Animal Production, and the American Dairy Science Association.

Mr. McMillan has been breeding Thoroughbred and Halfbred horses for more than a quarter of a century. Colts and fillies from the paddock of Steep Hollow Farm have appeared in the show ring, hunting field, and race track in many parts of the East and Canada.

He was a member of the Genesee Valley Breeders' Association and was a frequent exhibitor at the Annual Colt Show in Avon, and had been president of the Cornell Horse Show. Trophies and ribbons won over the years by his young stock there adorn the mantel of the McMillan home.

He is survived by his widow, Ruth Rice McMillan and three children, Donald R., East Lansing, Michigan, A. Thomas and Mrs. Edward Peterson of Ithaca; three sisters, Mrs. F. B. Chamberlin, Dundee, Mrs. E. S. Foster, Lansing, and Mrs. Charles Rogers of Wooster, Ohio.

Colorado News

The Colorado 100 Cow Club was organized and held its first meeting at Greeley, Colorado, on October 15, 1959. The Club has a potential membership of approximately 50. Four meetings have been held and attended by 25 to 35 of the potential members.

The organization of this group was brought about by a very rapid increase in the size of dairy operations in Colorado. It is the intention of this group to provide the opportunity for an exchange of ideas and information between dairymen, the Dairy Industry Department and Extension Service of Colorado State University, and other organizations which work closely with large commercial dairymen. It was also felt that present dairy organizations in the state were not designed to and could not meet the needs of this group in keeping up to date with technical dairy information. Discussions to date have covered dairy cattle nutrition, with emphasis on high energy rations and formulation of the lowest cost rations with current ingredient prices; the purchase of feed in large quantities; reproductive difficulties, and means of improving them; dairy cattle genetics and breeding programs for commercial dairymen; labor problems and discussions of large-scale dairying in the Los Angeles milk shed.

University of Connecticut Holds Career Day

A very successful Dairy Manufacturing Career Day was held at the University of Connecticut on October 22. Members of the Connecticut Association of Dairy and Food Sanitarians, under the chairmanship of E. I. KELLARSON, H. P. Hood and Sons, Hartford, Connecticut, recruited and transported 85 people who attended from high schools throughout the state.

The need for college-trained personnel in the dairy industry was emphasized by dairy industry speakers. Industry representatives appearing on the program included: J. J. EAGAN, Laboratory Director, Connecticut Milk Producers Association, Hartford; H. M. EWELL, Sales Representative, Pennsylvania Salt Manufacturing Company, Arlington, Massachusetts; R. M. PARRY, Chief of the Dairy Division, State Department of Agriculture, Hartford; and E. A. PERREGAUX, Director of Connecticut Milk for Health, Hartford.

The day included a luncheon through the courtesy of the Connecticut Association of Dairy and Food Sanitarans and the Connecticut Milk Dealers Association, a tour of the dairy manufacturing facilities, and attendance at the Connecticut-Boston University football game.

Animal Breeding Short Course

The University of Connecticut short course in Animal Breeding has been sheduled for January 30 to February 3, 1961, for those interested in any of the following classes of livestock: dairy cattle, beef cattle, sheep, swine, or horses.

This tuition-free course is open to anyone 18 yr. of age or over interested in physiology of reproduction, sterility problems, the basis of inheritance, selection systems, systems of breeding, and other related topics.

The purpose of this course is to give applicants a practical working knowledge of animal breeding principles and practices. Separate sections of the course will be developed where desirable for dairy cattle, meat animals, and horses. The course is particularly designed for owners, operators, managers, and herdsmen interested in learning more about animal breeding.

In addition to this course, the annual artificial insemination short course will be held at the University of Connecticut March 27–31. Students who wish to take this latter course are also advised to take the Animal Breeding Short Course in January.

Georgia News

The Dairy Science Club of the Dairy Department, University of Georgia, held its annual Fall Banquet on the evening of December 2, 1960, in the main Ballroom of the Georgia Center for Continuing Education.

Nineteen scholarships were awarded to the top undergraduates of the Dairy Department.

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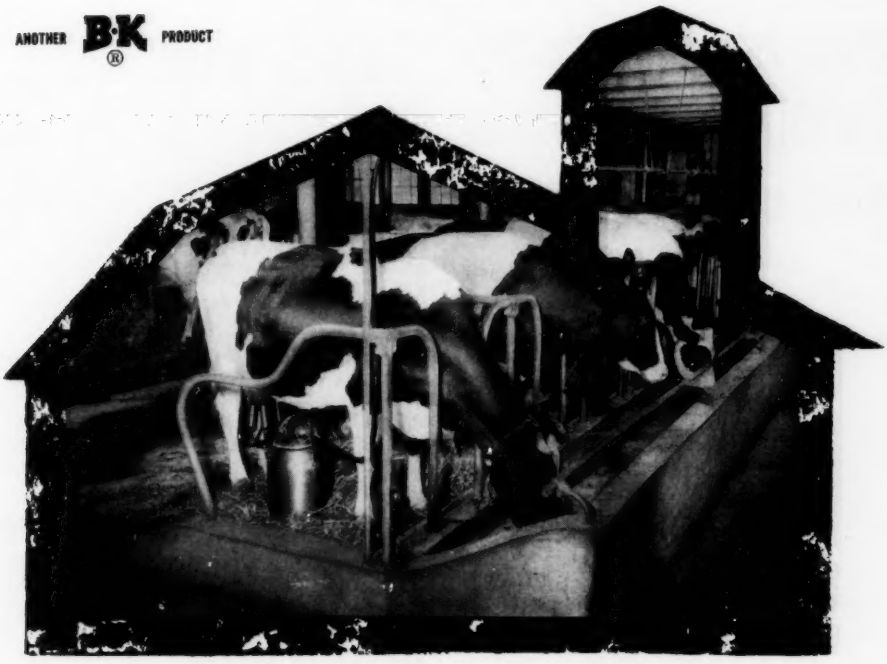

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These scholarships were donated by the dairy industry of Georgia.

The highlight of the evening was an address by R. E. HODGSON, Chief, Animal Industry Division, Agricultural Research Service, United States Department of Agriculture. Dr. Hodgson is an outstanding leader in the dairy industry, a very fine speaker, and is now President of the American Dairy Science Association.

Illinois News

Illinois Student Ranks First as Dairy Products Judge

J. W. HOCKING, a senior at the University of Illinois, took nearly all first-place individual awards in the 26th Collegiate Students' International Contest in Judging Dairy Products, held October 31, in Chicago, Illinois, concurrently with the 22nd Dairy Industries Exposition.

Illinois Winners of Collegiate Students' International Contest in Judging Dairy Products—J. K. Anniss, D. R. Yeazel, D. R. Henning, S. E. Seibert, Bonnie S. Houghtaling—American Dairy Princess, Harold Grange—Master of Ceremonies at Awards Banquet, J. W. Hocking, Joseph Tobias—Coach.

He placed first in judging of milk, cheese, ice cream, and all products. His performance allowed the three-man Illinois judging team to take first place in team standings, which carried with it a $2,300 cash fellowship for graduate study, sponsored by the Dairy Industries Supply Association.

In the team standings, University of Illinois' first place position allowed this team to retire from competition the All Products Bowl, which goes permanently to any team that wins it three times.

Twenty-five colleges and universities entered three-man teams of judges in the contest, which is sponsored annually by American Dairy Science Association and Dairy Industries Supply Association.

L. L. EWING received a Master's Degree in the Department of Dairy Science in September, 1960. The title of his thesis was Simplified Techniques for Maintaining Isolated Rabbit Testes by Fusion.

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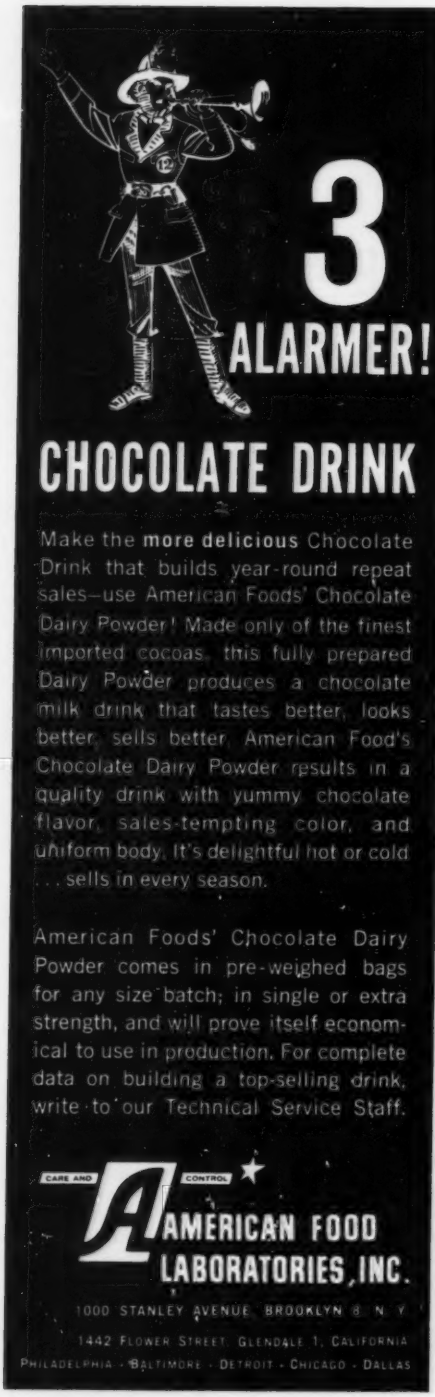
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Dr. William E. Sandine recently accepted a position as an assistant professor of Bacteriology in the Department of Bacteriology and Hygiene at Oregon State College, Corvallis, Oregon. Dr. Sandine was a research associate in the Department of Dairy Science at the University of Illinois for two years previous to accepting this appointment. While at Illinois he worked on lysine biosynthesis.

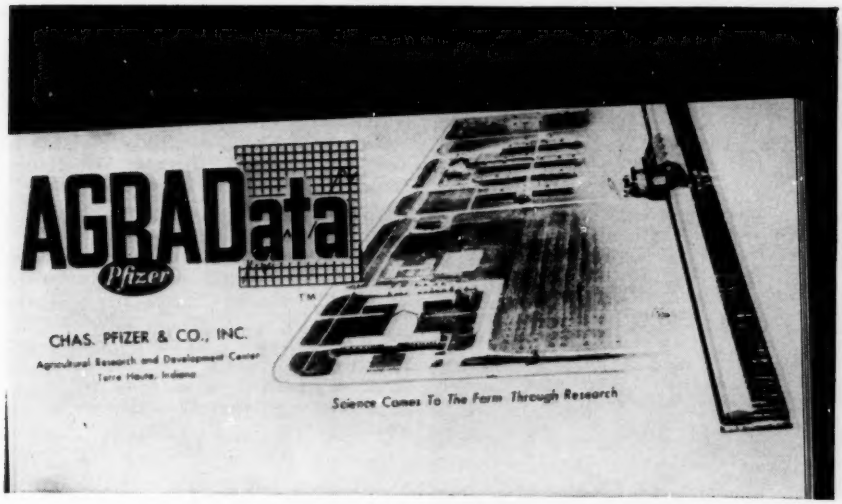
MAU-HUAL KUO recently accepted this position as research associate in the Department of Dairy Science at the University of Illinois. Dr. Kuo recently received a Ph.D. from the University of Michigan in the Department of Bacteriology.

Ohio State University

Approximately 220 alumni, guests, faculty, and students attended the 12th Annual Dairy Technology Homecoming Brunch, October 22, in the Auditorium of the Agricultural Administration Building. Feature events of the affair were the presentations of the Department of Dairy Technology Award of Merit to EARL GRIESER, president, Napoleon Creamery Company, Napoleon, Ohio, and the Certificate of Appreciation to C. J. LUDWIG, vice-president, Babcock Dairy Company, Toledo. Mr. Grieser was selected as Dairyman of the Year because of his outstanding contributions to the Dairy Industry at the national and state levels and for service to his community. Mr. Ludwig was honored for his contributions to the Department's student recruitment program. The presentations were made by ROY M. KOTTMAN, Dean of Agriculture, The Ohio State University.

Earl Grieser, president, Napoleon Creamery Company, Napoleon, Ohio (left), the 1960 recipient of the Ohio State University, Department of Dairy Technology Award of Merit, and Mrs. Grieser; and C. J. Ludwig, vice-president, Babcock Dairy Company, Toledo (right), recipient of the Department's Certificate of Appreciation, and Mrs. Ludwig.

Other highlights of the affair included the introduction of the classes of "ten" with T. D. HARMAN, assistant to the president, First National Bank, Circleville, representing the Class of '40; ED EBERT, production manager,



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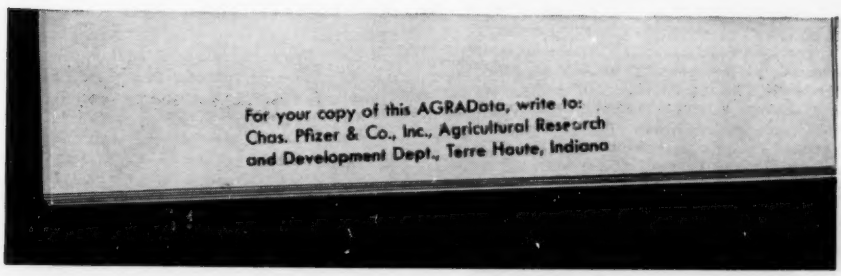
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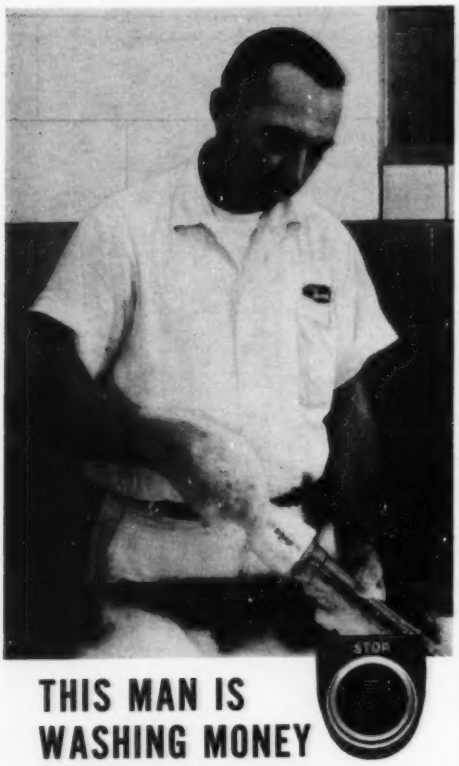

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Pickway Dairy, Circleville, the Class of '50; and P. L. CULLER, Creamery Package Company, Chicago, the Class of '60. Special recognition was given to MITSUGI SATO, president, Snow Brand Milk Products Company Ltd., Sapporo, Japan, M.S., '21, as the alumnus who travelled the farthest for the Brunch.

Food was served by members of the Dairy Tech Club. B. W. TAYLOR, '53, Dairy-Pak Butler, Inc., Columbus, served as MC of the affair.

I. A. GOULD, Chairman, The Department of Dairy Technology, The Ohio State University, returned November 1 from 3 mo. of absence for the purpose of conducting a survey for the United Nations Children's Fund on dairy training opportunities and needs. The survey involved visits to seven countries, with emphasis on the Near East: Iraq, Iran, India, and Pakistan.

Minnesota News

T. K. FETSCH, 5907 Hodgson road, St. Paul, has been awarded a $300 dairy husbandry scholarship for the academic year 1960-61. Fetsch is a sophomore in dairy production at the University of Minnesota's College of Agriculture, Forestry and Home Economics. The scholarship is provided by the Minnesota Dairy Industry committee to help and encourage worthy students majoring in dairy production.

International Dairy Products Judging Contest

The 26th International Collegiate Students Contest in judging dairy products was held in Chicago, Illinois, on October 31, 1960. The contest was co-sponsored by the American Dairy Science Association and the Dairy Science Association and the Dairy Industries Supply Association. Teams from 25 colleges and universities participated. The high individual in all products was J. W. HOCKING of the University of Illinois. Hocking's accomplishment was truly outstanding, in that he also won the contests in milk, cheese, and ice cream.

In addition to the unusual situation of having one individual win first place in three products, this contest was distinguished by the fact that three individuals tied for first place in butter, the remaining product. These individuals were R. G. CUNNINGHAM of Mississippi State University, W. P. SHERMAN of Iowa State University, and H. D. JOHNSON of the University of Minnesota. When flavor scores were used in an effort to break the tie, Johnson dropped to third place but Cunningham and Sherman remained tied for first.

The first three teams in all products were: 1. University of Illinois; 2. Iowa State University; 3. Oklahoma State University. The

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—ANSEL ESTELL, General Manager
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Columbus, Ohio

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—HOWARD FERREIRA
Dean Milk Company
Rockford, Ill.

This manual is being used regularly in our plant and we were surprised to get a report from our plant superintendent that he himself had gotten a great deal out of reading the manual, and that he is using it to check standard operating procedures throughout the plant. He remarked that he felt the book was outstanding in its detail, and yet that it was very easily read and extremely well organized.

—W. A. JOSEPHSON, General Superintendent
Turner Dairies, Inc.
Anniston, Alabama

CONTENTS

SECTION I states the objectives and requirements of the program.

SECTION II contains training schedules for all phases of plant operation for all types of plants. Many questions are asked for the trainee to answer before passing from one phase of training to the next, and progress reports and rating forms are provided.

SECTION III covers management development. Many study projects are outlined which are not only essential to management development but may result in savings to the plant that will more than pay the cost of training.

The Appendix contains a list of professional and trade organizations and reference reading publications.

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third-place team was tied, in score, by Texas A & M College, but the tie was broken on flavor scores. The three top teams received Fellowships valued at $2,300, $2,150, and $2,000 in order of placing.

Winning teams for each product were: Milk—University of Minnesota; Butter—Kansas State University; Cheese—University of Illinois; and Ice Cream—University of Connecticut.

The Committee on Judging

Dairy Products of the A.D.S.A., which works with this contest, is now headed by E. L. THOMAS, who is a former member of the Student Affiliate Committee and a former Editor of the Student News Section. He replaced G. M. TROUT, who had served in this capacity for a number of years.

STUDENT CHAPTER NEWS

J. E. JOHNSTON, Editor

A Section Devoted to News of Student Members

Message from the President, National Student Branch, A.D.S.A.

On behalf of the members of the National Student Branch of the American Dairy Science Association, I would like to express our sincere appreciation to each and every member of the senior organization who contributed to the building of the Student Branch section. The parent organization cleared the path of obstacles which might have caused a fledgling organization to falter and fail. Yet,

Delegates to Founding Convention

National Student Branch, A.D.S.A., 1960

Back row, left to right: E. Berousic, Oklahoma State University; Robert Bauer, Virginia Polytechnic Institute; Keith Jenkins, Ohio State University; J. E. Bertrand, Louisiana State University; Michael Bentivegna, Jr., Cornell University; D. K. Nelson, Colorado State University; Clyde Vair, Colorado State University; E. L. Jensen, Utah State University; G. A. Willardson, Utah State University. Middle row, left to right: P. M. Reaves, Advisor, Virginia Polytechnic Institute; R. L. Gilliland, Oklahoma State University; Duane Mattock, Penn State; R. J. Klausing, Ohio State University; R. E. Banderob, Montana State College; R. W. Ibbetson, Kansas State University; D. L. Dinger, University of Minnesota; John Healy, University of Minnesota; J. B. Mickle, Advisor, Oklahoma State University. Seated, left to right: Marvin Oetting, 3rd vice-president, University of Missouri; Joseph Lineweaver, 1st vice-president, Virginia Polytechnic Institute; Lewis Meyer, president, Michigan State University; Robert Van Pelt, 2nd vice-president, Washington State University; Francis Rothauge, secretary-treasurer, Oregon State College.

it left the Student Branch completely free to make its own decisions.

The purpose of the National Student Branch of the American Dairy Science Association is to provide benefits to students which are not available to them otherwise. The sincere hope of the Executive Board is that service will be the guiding light of our branch of the Association. Indivilually, we might do little, but joining together, we can do much. As students today, we trust that in a few short years we may be able to introduce new techniques and introductions comparable with leaders of the past. The primary prerequisite is work. To this we dedicate ourselves.

Our organization is new. We have not been tried by the test of time, nor do we have the experience that this test brings. When we are each given our chance to show our worth, let it be said that each member of the Student Branch went the second mile.

Realizing that the interests of our student members cover a broad spectrum of dairy subjects, our goal is to satisfy a variety of needs. Our goal is not to compete with the existing organization, but to provide services, now unavailable, geared to the needs of the student affiliate. Starting at our next annual meeting, we hope to do this by introducing to our members:

1. The men who make our Association. Providing facilities to have lunch and talk with outstanding leaders of the dairy world.
2. Career opportunities. Presenting outstanding speakers from the various fields, discussing the advantages and disadvantages of their fields of endeavor, as well as the preparation required by that industry.
3. Outstanding chapter activities. Presenting to the group a summary of highlights of the chapters which are unique and might be incorporated into other chapters.
4. A means of communication. Providing through the JOURNAL OF DAIRY SCIENCE and the annual meetings of the Student Branch various ways to solve common problems.

This then, is our goal—to provide services not attainable by individuals or individual chapters. By working together, we will have an organization which is greater then the sum of its parts.

LEW MEYER, President
National Student Branch
A.D.S.A.

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News from the Eastern Utilization Research and Development Division, USDA

New discoveries and methods of protein research were discussed October 25 and 26 in a joint conference of about 80 scientists from the U. S. Department of Agriculture, state experiment stations, and universities. The conclave was held at the Agricultural Research Service's Eastern Utilization Research and Development Division in Wyndmoor, Pennsylvania (near Philadelphia), and was presided over by P. A. WELLS, director of the division.

One of the featured speakers was WALTER KAUZMANN, of Princeton University, who described important changes in the union between water and proteins and the volume changes involved. Another was CHARLES TANFORD, of Duke University, who demonstrated that the measurement of the electrical charge on proteins is an important way of revealing the properties of milk proteins.

W. G. GORDON, of the Eastern Utilization Research and Development Division, showed for the first time, by skillful analyses on the proteins of milk from individual cows, that the amounts of some of the amino acids present varied slightly. The discovery had been made in England 6 yr. ago that the proteins of the milk from individual cows differed, but the reason for the difference was unknown. This finding is of great interest to protein chemists, although it does not affect the value of milk as a food.

Industry News

F. N. FORD has been promoted to manager of the Meadow Gold Dairies plant in Huntsville, Alabama. A veteran of 9 yr. experience with Beatrice Foods, he has been office manager of Tro-Fe Dairy Co., Gadsden, Ala., since 1951.

F. N. Ford

A native of Gadsden, Ford is a veteran of 6 yr. service in the U. S. Army, with overseas duty in the European theater during World War II. He entered as a private in 1940 and held the rank of major at the time of his discharge. His college training and business experience primarily have been in the field of business administration.

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milk, ice cream products, butter, cheese, and other milk by-products. It will function under the name of Empresas Lacteas Foremost, S.A., whose president is A. Q. MEZA. The company organization will include MAX BLOCH and R. Q. MEZA, together with other important local business people in conjunction with Foremost. Bloch and A. Q. Meza have operated dairy companies in San Salvador for many years. They will be active in the administration of the new company. A complete line of dairy products will be offered to the public under the Foremost brand name.

Dairy Technology Societies

Central Illinois—Joseph Tobias, Department of Food Technology, University of Illinois, Urbana, was the featured speaker at the group's November 9 meeting at the Figure 8, Decatur. His subject: The Ice Cream Standards and Labeling Regulations.

Central Michigan—The November meeting had Bernard Schweigert as guest speaker, on the topic Educational and Industrial Team Work in the Food Industry. Michigan State University's Dairy Products Judging Team were also guests, and reported on their judging contest experiences, particularly on the recent Collegiate Students' International Contest in Chicago.

Detroit—November 14 meeting had B. S. Schweigert as speaker. His topic: Educational and Industrial Team Work in the Food Industry.

Kansas City Area—November 17 meeting was dedicated to plant managers. Featured talk was by H. L. Mitten, Jr., director of technical sales, The Creamery Package Manufacturing Company, Chicago—Processing Trends Shown by the 1960 Dairy Industries Exposition.

Metropolitan—Effective Supervisory Management was the title of W. R. Cox's talk at the November meeting. Cox is manager, Personnel Development Dept., Dairymen's League Co-op. Association, Inc.

North Carolina—F. M. Gray, director of production, Sealtest Foods, Inc., Southern Division, Charlotte, North Carolina, spoke at the November 9 meeting, on Practical Knowledge Obtained Concerning Weight and Measure Problems.

Ohio—November meeting of the four Ohio societies was designated High School Night. Members and their invited guests toured a major dairy operation in the respective areas. I. A. Gould, chairman, the Department of Dairy Technology, The Ohio State University, spoke on Some Challenges of Our Industry, at the Northeastern Ohio and Maumee Valley societies. At the Central Ohio and Cincinnati societies, an animated slide sequence was shown, illustrating the Dairy Technology curriculum at Ohio State and the opportunities in the Dairy Industry.

Tri-Cities—At the dinner meeting at the Hillbrook Restaurant, November 8, H. E. Calbert, professor of Dairy and Food Industries, University of Wisconsin, spoke on Are Our Quality Tests Telling Us What We Want to Know?

Tri-State—Harry Katz, head entomologist, Eleo Manufacturing Company, Pittsburgh, Pennsylvania, spoke on Animals, Insects, and You at the November 16 meeting. There was also a panel discussion on bulk handling.

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BINDING OF RIBOFLAVIN AND RIBOFLAVIN PHOSPHATE BY THE PROTEINS OF MILK

ABRAHAM LEVITON AND MICHAEL J. PALLANSCH
Dairy Products Laboratory, Eastern Utilization Research and Development Division, USDA
Washington 25, D. C.

SUMMARY

The binding of riboflavin and riboflavin phosphate by the proteins of milk, particularly by α- and β-casein, has been studied. Binding constants have been determined and, from these, thermodynamic quantities have been calculated. The binding is of a low order of magnitude with an unusually high temperature coefficient. The binding by the calcium caseinate–phosphate complex in milk is equivalent to the binding by its component protein molecules. Certain similarities between the binding by the caseins, and the binding by flavoprotein enzymes, have been noted. The data indicate strongly that phenoxyl residues constitute the loci of binding.

As part of a program devoted to the study of the effect of additives on coagulation in milk it became a point of interest to know whether the calcium caseinate–phosphate complex in milk possessed solubilizing or binding characteristics peculiar to its micellar structure and distinct from the binding characteristics of its component molecules. On a priori grounds it can not be assumed that equivalence exists, for by analogy with the extraordinary solubilizing properties shown by detergent micelles, the so-called protein micelles conceivably may also have extraordinary binding characteristics. Conversely, it is conceivable, too, that the micelle may bind less substrate than its component molecules.

Riboflavin and its phosphate were selected as substrates for this study for a number of reasons. Riboflavin is a neutral molecule and its binding would not necessarily disturb the calcium caseinate–phosphate complex. A number of phenomena encountered in dairy technology are or may conceivably be related to the binding of riboflavin or riboflavin phosphates. Such for example are: (a) the photosensitizing action of riboflavin in the decomposition of ascorbic acid, and the formation of methional from methionine; (b) the partition of riboflavin between curds and whey in the manufacture of casein and cheese; and (c) the behavior of riboflavin phosphate in prolonging the time required to bring about thermal coagulation of the proteins in milk.

There are also points of general interest in studies on the binding of riboflavin and riboflavin phosphate. These pertain to the nature and number of the binding sites; to the relative binding effectiveness of various milk proteins; to the influence of the anionic phosphate group in riboflavin phosphate on binding and, finally, to the similarities which may exist between the general binding of riboflavin phosphate by proteins and the highly selective binding of riboflavin derivatives in enzyme systems.

Received for publication June 18, 1960.

EXPERIMENTAL PROCEDURE

Materials. α- and β-Casein were isolated from skimmilk by the method of Hipp *et al.* (5). This method depends on the difference in solubility in aqueous urea. α-Casein was found to be electrophoretically pure at pH 8.6. β-Casein contained 5% α-casein as an impurity. Electrophoretically pure β-casein was not realized with the urea method. The removal of the 5% α-casein impurity, however, was effected by the method of Warner (15), based on the different solubilities of these proteins in aqueous solutions at low temperatures. α-Casein prepared by the urea method dissolved in NaOH to yield turbid solutions. The particles responsible for the turbidity were removed. The α-casein preparation dissolved by means of borax in a 50% alcoholic solution, 0.15 *M* in NaCl, was fractionally precipitated with alcohol. The large particles precipitated first and, following their removal by filtration, the main body of α-casein was precipitated from the filtrate by the addition of water and acid. β-Lactoglobulin prepared according to Palmer [see Gordon *et al.* (4)] was recrystallized four times. Bovine serum albumin was a commercial product which had been recrystallized twice.

Riboflavin of U.S.P. quality was recrystallized in the manner prescribed by Pasternack and Brown (11). Riboflavin -5′-monosodium phosphate dihydrate, a Hoffmann-LaRoche product,[1] in paper electrophoresis exhibited two faint bands in addition to the major band belonging to the phosphate.

Methods. Equilibrium dialysis furnished data for the calculation of moles solute bound per 10^5 g. protein (12). The 7.5-ml. protein solution within the cellophane bag contained 0.188 g. protein. The bag was submerged in 7.5 ml. of either a buffer or salt solution contained in a screw-capped vial. A glass marble within the bag facilitated mixing during the slow end-over-end motion of the vial during a three-day equilibration period, usually at 4°. The bags, following loading with sample, were carefully tied so that the membranes were approximately under the same tension, and the presence of air bubbles was minimized. This done, the weighing of the bag before and after dialysis permitted conclusions to be drawn in certain instances concerning the manner in which the number-average particle weight of the protein was affected by changes in temperature and solution composition. Binding data pertaining to riboflavin phosphate required the application of a correction factor to compensate for the uneven distribution of ions brought about by the Donnan effect.

The fully developed equation based on Donnan ratios for the calculation of the correction factor requires knowledge of the volumes of the equilibrated solutions inside and outside the bag. However, approximation equations in which these volumes do not appear explicitly may be used to advantage (12).

Basically, the full equation permitted the calculation of the concentration of positive ions inside and outside the bag, thus:

[1] It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

$$m' = \sum \frac{(M-m)^{\nu_r}\ \nu_r\ M_r}{(M-m')^{\nu_r} + (P+m')^{\nu_r}\left(\frac{Va}{Vi}\right)^{\nu_r - 1}}$$

M—total moles of cations associated with buffer salts and protein
m′—total moles of cation within the bag
P—equivalents of protein within the bag
ν_r—valence of anion of species r
V_a, V_i—volumes of solution outside and inside bag, respectively
M_r—total moles of anion of species r

The summation is taken over all anionic species present in the system. The equation may be solved graphically for m′, and the cationic concentration inside and outside the bag may then be calculated. This done, the equilibrium riboflavin phosphate concentration may be calculated from the Donnan ratios (2).

Dialysis experiments conducted at 20° C. required the employment of aseptic techniques. Protein and riboflavin solutions were sterilized by filtration; and membranes, thread, pipettes, and implements were sterilized by steam.

Concentrations of riboflavin and riboflavin phosphate were determined by absorbance measurements at 445 mμ. The magnitude of the error at the lowest concentration was of the order 0.5%; the difference in concentration was about 30%.

In experiments to determine the binding by the protein complex of milk, milk was dialyzed against its dialyzable constituents (prepared by dialyzing water against a large excess of milk), supplemented either with riboflavin or riboflavin phosphate. Casein was determined according to the method recommended by the A.O.A.C. (10).

Phosphate buffer solutions were prepared from K_2HPO_4 of analytical grade. pH determinations were made by means of a glass electrode. In studies pertaining to the effects of copper, zinc, iron, and molybdenum, the outside solutions were adjusted to contain one of the following salts: $CuSO_4$, $ZnSO_4$, $FeCl_3$, and $(NH_4)_2MoO_3$ in 0.001 *M* concentration. In studies on the effect of Ca^{++}, the protein solutions were adjusted to contain 0.005 mole $CaCl_2$ per liter. All solutions were made 0.4 *M* with respect to $NaNO_3$. To study the effect of urea, both the protein and outside solutions were adjusted to contain 6.6 *M* urea and 0.1 *M* phosphate buffer of pH 6.75.

The binding isotherm for riboflavin. Table 1 shows how the binding of riboflavin by α- and β-casein in phosphate buffer of pH 6.75 and ionic strength 0.2 varied with equilibrium concentration. A straight line passing through the origin defined the relationship with sufficient exactness in the concentration range specified in the table. The constant of proportionality may be designated either as an apparent association constant or as a partition coefficient.

The magnitude of the binding is quite small. The binding by α- and β-casein, respectively, at an ionic strength of 0.2, was characterized by association constants, the values of which were 1.7 and 1.3×10^3 liters per 10^5 g.

Influence of temperature on the association constant. Table 2 shows how the binding of riboflavin decreased two- to threefold as the temperature was

TABLE 1

Binding of riboflavin by α- and β-casein in phosphate buffer

Protein	Equil. concn. riboflavin [R] moles per liter × 10^5	Riboflavin bound, r moles per 10^5 g.	$\frac{r}{[R]}$ liters per 10^5 g.
α-casein	1.7	0.029	1,700
	2.3	0.039	1,700
	3.5	0.060	1,720
	6.2	0.110	1,770
	6.2	0.105	1,690
	8.7	0.152	1,740
β-casein	2.0	0.026	1,300
	2.7	0.034	1,260
	3.9	0.050	1,290
	6.7	0.086	1,280
	9.4	0.122	1,300

pH—6.75.
Ionic strength—0.2.

raised from 4 to 55° C. The association constant belonging to β-casein, for example, decreased from 1.3×10^3 liters per 10^5 g. at 4° to 4.7×10^2 liters per 10^5 g. at 55° C. Knowledge of the values of the association constant at different temperatures permitted the calculation of the change in enthalpy accompanying the binding. Change in enthalpy, $\triangle H$, was not constant over the temperature range 4 to 55°, but varied in some manner with temperature. In the absence of sufficient data to define this relationship, it has been assumed that $\triangle H$ is independent of temperature in the temperature range 4 to 20°, and accordingly the values of $\triangle H$ belonging to α- and β-casein in this temperature range have been calculated. Much the same assumption is made in the calculation of the values of $\triangle H$ which appear in the literature and, hence, a basis for comparison exists. $\triangle H$ values of −5.8 and −4.8 kcal. per mole were obtained, respectively, for the binding by α- and β-casein. From the value of the association constant for β-casein, with a molecular weight of 25,000, the change in free energy at 4° of −3.1 kcal. per mole, and the change in entropy of 6 cal. per mole per degree, was calculated. As the temperature was raised from 20 to 55°, the decrease in the values of k was less than one would anticipate

TABLE 2

Variation in the value of the association constant belonging to the casein-riboflavin complex with variations in temperature

Protein	α-Casein			β-Casein		
Temp. ° C.	Association const. K *(liters per 10^5 g.)*	Av. enthalpy change (−△H) *(cal. per mole)*	Osmosis [a] index *(g.)*	Association const. K *(liters per 10^5 g.)*	Av. enthalpy change (−△H) *(cal. per mole)*	Osmosis index *(g.)*
4	1,700		+0.4	1,300		+1.1
		5,800			4,800	
20	950			860		
55	820		+1.1	570		+0.4

[a] Osmosis index—algebraic difference of increase in weight of bag containing no protein, and of bag containing protein.

had $\triangle H$ remained constant. The indices given in the last column relating to the quantity of water diffusing through the dialyzer membrane as the result of osmosis showed that the number of α-casein particles increased and the number of β-casein particles decreased as the temperature was raised from 4 to 55°. This change in the state of aggregation with changes in temperature could conceivably influence the binding in the direction indicated by the data.

The influence of ionic strength, pH, metals, and urea on the association constant. The association constant for the binding of riboflavin by both α- and β-casein in phosphate buffer, pH 6.75, was not influenced by changes in ionic strength from 0.1 to 0.5. The values of the association constant changed slightly with a change in pH from 6.2 to 7.7. No mediating effect was observed for cations such as Cu^{++}, Fe^{+++}, and Zn^{++}, which form chelates with riboflavin and complexes with α- and β-casein. A slight competitive effect of Cu^{++} and Zn^{++} was observed in β-casein solutions. No mediating effect was observed for either calcium or molybdenum.

Binding of riboflavin was seriously weakened in the presence of urea. The association constant for the binding by α-casein in 6.6 *M* urea solution was 180 liters per 10^5 g.—a value approximately 10% of that observed in the absence of urea. Similarly, the value of association constant for the binding of β-casein in 6.6 *M* urea was approximately 6% of that observed in the absence of urea.

The indices relating to osmosis showed a strong disaggregation effect of urea on α- but not on β-casein. This is consistent with observations reported in the literature to the effect that β-casein is monodisperse in neutral aqueous solutions and that α-casein is polydisperse (15).

Binding of riboflavin by the calcium caseinate—calcium phosphate complex in milk. In Figure 1, two curves are compared—one representing the binding of riboflavin by a mixture containing four parts of sodium α-caseinate and one part sodium β-caseinate, and the other representing the binding by the calcium caseinate–calcium phosphate complex in milk. The quantity of bound riboflavin was approximately the same in the two systems, showing that neither the micellar structure nor bound calcium mediated the binding. The failure of calcium to mediate the binding has already been indicated. The complex in milk is said to be associated with colloidal $Ca_3(PO_4)_2$; it may easily be shown experimentally that $Ca_3(PO_4)_2$ does not bind riboflavin.

The binding of riboflavin phosphate by α- and β-casein. The data pertaining to the binding of riboflavin phosphate contain an element of error, in that a large correction has been applied to compensate for the uneven distribution of anions brought about by the Donnan effect. In view of this, the data are significant only with respect to the order of magnitude of the values involved. The much greater solubility of riboflavin phosphate compared with riboflavin permitted the extension of the binding data to much greater equilibrium concentrations and, hence, a more complete characterization of the binding curve was realized.

In Figure 2, points representing the ratios between riboflavin phosphate bound per 10^5 g. and the equilibrium concentration were plotted as ordinates,

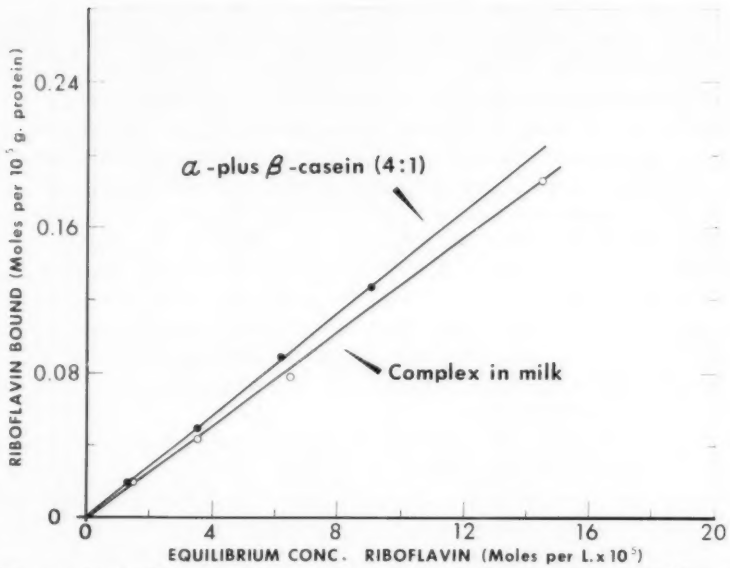

FIG. 1. The binding of riboflavin by the calcium caseinate–calcium phosphate complex in milk, and by a solution containing sodium α- and β-caseinates in the ratio 4: 1.

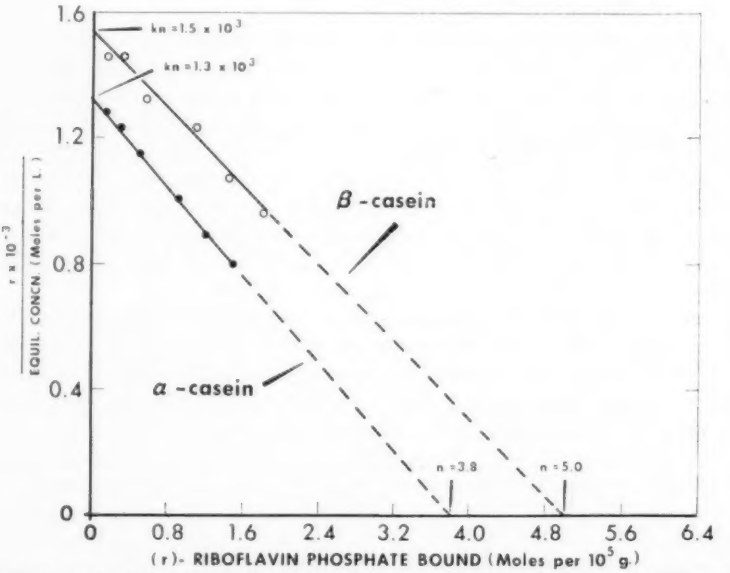

FIG. 2. The binding of riboflavin phosphate by α- and β-casein in phosphate buffer pH 6.75, ionic strength 0.2.

and points representing moles bound were plotted as abscissas. Reasonably good lines permitting extrapolation were drawn through the points. The lines belonging to β-casein and α-casein, respectively, intersected the abscissa axis at points representing a maximum of five moles riboflavin bound per 10^5 g. β-casein and about four moles bound per 10^5 g. α-casein.

The lines belonging to α- and β-casein intersected the ordinate axis at points representing the respective apparent association constants, 1,300 and 1,500 liters per 10^5 g.

The effect of temperature on the binding of riboflavin phosphate. Table 3 shows how the apparent association constant for the binding of riboflavin phosphate varied with temperature. The rate at which the association constant

TABLE 3

Influence of temperature on the binding of riboflavin prosphate by α- and β-casein in phosphate buffer, pH 6.75, ionic strength 0.2

Protein	α-Casein		β-Casein	
Temp. ° C.	Association const. *(liters per 10^5 g.)*	Enthalpy change ($-\Delta H$) *(cal. per mole)*	Association const. *(liters per 10^5 g.)*	Enthalpy change ($-\Delta H$) *(cal. per mole)*
4	1,300	4,900	1,500	7,700
20	800		700	
55	450		350	

changed with temperature had the same magnitude as the rate of change observed for the binding of riboflavin.

The binding of riboflavin and riboflavin phosphate by various proteins. In Table 4, the association constants are given for the binding of riboflavin phosphate by α- and β-casein, native and denatured bovine serum albumin, and native and denatured β-lactoglobulin. The following order with respect to binding affinities was observed: α-casein, β-casein or bovine serum albumin, β-lactoglobulin.

Denaturation effected a significant increase in the binding capacity of β-lactoglobulin. Thus, a certain openness in structure was required for maximum binding efficiency.

TABLE 4

Binding of riboflavin and riboflavin phosphate by various proteins at pH 6.75, ionic strength 0.2

Protein	Association const. (riboflavin) *(liters per 10^5 g.)*	Association const. (riboflavin phosphate) *(liters per 10^5 g.)*
α-casein	1,700	1,300
β-casein	1,300	1,500
Bovine serum albumin	1,000	1,100
Bovine serum albumin (denatured)	1,200	1,200
β-lactoglobulin	300	100
β-lactoglobulin (denatured)	1,100	300

The influence of metals and ionic strength on the binding of riboflavin phosphate. As in the binding of riboflavin, Ca^{++}, Cu^{++}, Zn^{++}, Fe^{+++}, and Mo^{++} failed to mediate the binding. Pertinent data are omitted because of their negative character.

In the range of ionic strength 0.5 to 1.0, a range in which the Donnan effect is largely suppressed, no significant competitive effect on the part of buffer anions was observed. α-Casein but not β-casein was effectively salted out of phosphate buffer solutions at pH 6.75 and ionic strength 1.0.

The binding of riboflavin phosphate by the calcium caseinate–calcium phosphate complex in milk. Binding data, obtained in the equilibrium dialysis of skimmilk against its diffusable constituents augmented with riboflavin phosphate, would appear superficially to indicate that the percentage of riboflavin phosphate bound by the complex is greater than the percentage bound by an equivalent weight of the sodium caseinates at pH 6.75. However, the Donnan effect is reduced in milk because of the binding of Ca^{++}. Application of the Donnan correction showed that the apparent association constants were the same: 1,400 liters per 10^5 g. In calculating the Donnan correction the ionic strength of milk was taken as 0.075 (17), and it was assumed that 1.04% calcium was bound by casein (1).

DISCUSSION

Knowledge of the composition of the calcium caseinate–phosphate complex in milk and the binding constants of its component proteins permits the calculation of the quantity of riboflavin or riboflavin phosphate bound by the complex. The complex in milk is a loose, easily penetrated aggregate with no characteristic binding attributes such as a micellar or globular structure conceivably might possess. Electron microscopy discloses the complex as individual, spherical particles. Because the resolving power of the electron microscope is not great enough, fine structure is not revealed. The data on binding, serving to supplement the information obtained with the electron microscope, show that the micelle in milk is sufficiently porous to invite into its interior large neutral organic compounds.

The complex, although globular in appearance, behaves as a moiety of extended particles. Fine structure rather than shape is the feature which distinguishes one form of a binding entity from another. Thus, β-lactoglobulin in its globular undenatured form binds only insignificant amounts of riboflavin; the extended denatured form, however, binds to the same degree as bovine serum albumin and β-casein.

Because of its low solubility, riboflavin is available for binding studies only in a limited concentration range. A unique interpretation of binding data is, therefore, not possible. The data lend themselves to interpretation with a minimum degree of bias, either in terms of the participation of a small number of identical binding sites with small association constants, or in terms of solution theory as a distribution of riboflavin between two phases—an aqueous and a protein phase. The interpretation in terms of identical binding sites will be considered in another portion of this discussion. Salting out of α-casein is not

attended by any significant salting-out or salting-in of riboflavin or its phosphate. The binding of these solutes has, therefore, a phase distribution character; that is, each protein molecule as it binds riboflavin and riboflavin phosphate behaves as if it were a part of a separate phase.

Certain aspects of anion binding are not observed in the binding of riboflavin and its phosphate. The association constants for the binding of riboflavin are of the same order of magnitude as that observed by Klotz and Walker (9) in the binding of sulfanilamide by bovine serum albumin. The pKa values are approximately the same, justifying the conclusion superficially that it is the anion of riboflavin which is bound. This conforms with the conclusions of Klotz and Walker concerning the influence of pKa on the binding of sulfonamides. However, there are significant points of difference between the binding of riboflavin and the binding of anions. The order with respect to the affinity of the various proteins for riboflavin is quite different from the order (8) with respect to their affinity for anions (see Table 4). The large enthalpy change (Table 2 and 3) connected with the binding of riboflavin and its phosphate is atypical of anion binding (6). Failure of the doubly charged phosphate group in riboflavin phosphate to contribute substantially to the degree of binding (Table 3) and absence of competition between buffer anions and riboflavin phosphate are also atypical of anion binding.

Although the order with respect to the affinity of native milk proteins for riboflavin is essentially the same as the order with respect to the binding of Cu^{++} (3), no evidence was obtained of trace metal mediation in the binding of riboflavin and its phosphate.

Urea in large concentrations displaced riboflavin from both α- and β-caseins, but displacement surprisingly was not complete even in 6.6 *M* urea solutions. Klotz, Triwush, and Walker (7) have shown that the displacement of the strongly bound anionic dye methyl orange from serum albumin is measurably complete in 6.6 *M* urea.

The binding data lend themselves to a reasonable interpretation based on the presence in each of the various proteins of a single set of identical binding sites. The selection of these binding sites is based on the hypothesis that the ureide-carbonyl, and the aromatic-amine groupings of the riboflavin molecule, will seek out preferentially the most complementary groups on the protein molecule. This condition is met if the phenoxyl, that is, the tyrosine residues, are the loci of binding. This residue contains a hydrogen-donor hydroxyl group attractive to the ureide-carbonyl group of riboflavin, and the residue as a whole is attractive to the aromatic-amine grouping.

The respective number of phenoxyl groups (see Table 5) present in 10^5 g. of α-casein, β-casein, bovine serum albumin, and β-lactoglobulin (14) is 45, 18, 28, and 20, respectively. The order with respect to affinities (Table 4) is, therefore, not the same as the order with respect to the number of phenoxyl groups. Additional assumptions are required. It is doubtful that all of the phenoxyl groups are free to bind riboflavin; interaction between the hydroxyl and the combined carboxyl and cationic groups on the protein molecule may

TABLE 5
Calculated ordar of relative affinity of various proteins for riboflavin based on free phenoxyl groups

Protein	Cations	Anions	Phenoxyl	Phenoxyl (free)
α-casein	104	102	45	11.0
β-casein	84	81	18	5.0
Bovine serum albumin	148	139	28	5.0
β-lactoglobulin	104	142	20	4.0

take place. Klotz and Urquhart (8), in their attempt to establish a configurational basis for anion binding affinities, considered this internal interaction. An approximation to the number of free phenoxyl groups can be made on the basis of the following reasonable assumptions:

(a) hydrogen is bonded with the same intensity to anionic and cationic groups (small differences would complicate the mathematical treatment without altering conclusions);
(b) internal binding is governed by the laws of mass action; and
(c) there is an average of five phenoxyl groups per 10^5 g. of β-casein which are free to bind riboflavin (see Figure 2).

Based on these assumptions, the following relationships may be derived, thus:

$$N_{free} = \frac{N_{total}}{k \triangle N + 1}$$

$$k = \frac{N_{total} - 5}{5 \triangle N} = 0.016$$

N_{free} is the number of free phenoxyl residues, N_{total} the total number, $\triangle N$ the difference between the sum of the number of cationic and anionic groups and the number of phenoxyl groups, and k is an affinity constant common to all proteins. The values in the last column of Table 5 show the calculated number of phenoxyl residues free to bind riboflavin, and it is apparent that the order with respect to these binding sites is essentially the same as the order of binding affinities observed experimentally. Not only is there qualitative agreement but the relative indices agree quite well with the relative maximum values of the association constants. With employment of strong urea solutions as solvent the ratio between the association constants belonging to α- and β-casein approaches quite closely the ratio of 2.2:1 between the free phenoxyl groups. This observation may mean that the binding sites in the polymeric form of α-casein are not as available as they are in the monomeric form.

Some comment is apropos concerning the failure of the phosphate grouping to contribute significantly to the binding. Theorell and Nygaard (12) have shown that the association constants for the binding of riboflavin and riboflavin phosphate, respectively, by Warburg's yellow apoenzyme are 10^2 liters per mole and 10^8 liters per mole. Thus, the order of magnitude of the constant for the binding of riboflavin is the same as that observed in the binding of riboflavin by milk proteins. The order of magnitude, however, for the binding of riboflavin phosphate is about 10^6 times greater. Theorell and Nygaard have postulated, in view of their observation, that only the doubly charged phosphoric

acid residue is reactive, that firm binding of riboflavin phosphate is possible only under conditions in which two positively charged groups on the protein molecule are in close proximity. This reasoning applied to the data on milk proteins leads to the conclusion that, in these proteins, two positively charged groups would not be found in juxtaposition with each other and with a tyrosine residue.

The large value of $-\triangle H$, 11,700 cal. per mole, observed in the enzymic binding of riboflavin phosphate, may be presumed to represent a nonionic contribution to binding inasmuch as in typical anion binding $-\triangle H$ is very small (6). Hence, it is a matter of interest to compare the thermodynamic data pertaining to the riboflavin–β-casein system with data on the riboflavin phosphate–apoenzyme complex. The value of $-\triangle H$, 4,800 cal. per mole, compared with the value 11,700 cal. per mole associated with the binding in Warburg's yellow enzyme, suggests that in the enzyme at least two tyrosine residues are cooperatively binding riboflavin. The change in entropy, which is positive in both the riboflavin, β-casein, and the riboflavin phosphate, apoenzyme systems, about 6 and 4 cal. per mole per degree, respectively, also points to a resemblance in the character of the interactions.

Although the magnitude of the binding by milk proteins is quite small, the quantity of riboflavin bound at low temperatures in milk comprises 25-30% of the total riboflavin. The partition of riboflavin between curds and whey in the manufacture of various products would be determined by the relationships presented in this paper. The employment of relatively high temperatures would favor the retention of riboflavin in milk serum. In a subsequent paper it will be shown that the photochemical decomposition of riboflavin is inhibited to some extent by casein, and that the degree of inhibition may be related to the degree of binding.

Attractive as the mechanism based on the participation of a small number of identical binding sites appears to be, the data presented in this paper do not effectively rule out other mechanisms. Viewing, for example, the binding of riboflavin phosphate as a partition of the phosphate between two phases, one may postulate that the decrease in the value of the partition coefficient at the higher concentration levels is an indication of changes in the activity coefficient of the phosphate brought about by dimerization.

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ACTION OF RENNIN AND PEPSIN ON β-CASEIN: INSOLUBLE AND SOLUBLE PRODUCTS

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SUMMARY

β-Casein was hydrolyzed to a considerable extent by the enzymes pepsin and rennin at pH 6.4. Pepsin hydrolyzed β-casein more extensively than rennin when given sufficient time. Hydrolysis of β-casein was evidenced by an increase in solubility at pH 4.7, and an increase in solubility in 2% trichloroacetic acid (TCA). Solubility in 12% TCA was about the same before and after enzyme treatment; hence, it was not a suitable reagent for demonstrating hydrolysis. The insoluble portion (pH 4.7; 2% TCA) of β-casein after enzyme treatment was heterogeneous, containing at least three components. The soluble fraction was very heterogeneous and contained ten or more components. The major products released by rennin and pepsin have similar electrophoretic behavior, but there were differences among the minor products.

Reports on the action of the enzymes rennin and pepsin on β-casein have been diverse. Some have concluded that only α-casein is altered by rennin, whereas β-casein and γ-casein are not attacked (3, 1), but are merely coprecipitated unchanged along with the altered α-casein in the presence of calcium salts (3). Others have reported that β-casein shows only a slow general proteolysis (6, 7); that the prolonged action of rennin on β-casein gives a product no longer precipitable with calcium ion (9); and that β-casein is split into two components in 4 hr. by rennin (8). A recent report has shown that β-casein is solubilized considerably by pepsin in 5 min. (10).

The present investigation was undertaken to learn more about the action of the enzymes rennin and pepsin on β-casein, and to clarify the reports just cited. Since some of the conflict appeared to be due to the choice of conditions for precipitating the treated β-casein, precipitation at pH 4.7 as well as precipitation with two concentrations (2 and 12%) of trichloroacetic acid (TCA) were compared. The components of the insoluble and soluble fractions were investigated at the same time by electrophoresis and chromatography.

EXPERIMENTAL PROCEDURE

Enzymes. Pepsin was a crystalline, commercial product. Rennin was a highly purified product obtained through the courtesy of Dr. R. A. Sullivan, National Dairy Research Laboratories, L. I., N. Y. This rennin had the same specific activity as crystalline rennin.

β-Casein was prepared by the method of Hipp *et al.* (5), utilizing differential solubility in aqueous urea solutions.

Preparation of dry fractions. β-Casein and all precipitates were dried by

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washing with acetone and ether and removing the solvent in a stream of air. Solutions were concentrated and dried by a rapid, large-surface vacuum technique (4) at 30-35° C.

It was observed that all washings in all experiments, after evaporation of ether, left some yellowish, fat-like residue. These residues were not analyzed further.

Enzyme reaction. A 2% β-casein solution was prepared by dissolving 5 g. of dry protein in a mixture of 2.5 ml. of 1 *N* NaOH and 240 ml. of distilled water, and the solution was adjusted to pH 6.4. Five milligrams of enzyme was dissolved in 5 ml. of water and added to the casein solution at 30° C. The reaction was continued for a definite time at 30° C. The enzyme reaction was stopped either with TCA or heat, according to the type of experiment being run.

(a) *pH 4.7 precipitation.* The enzyme reaction was stopped by placing the flask in a boiling water bath and stirring for 15 min. The contents of the flask were quickly cooled to room temperature, adjusted to pH 4.7 with 0.1 *N* HCl, and centrifuged. Nitrogen and phosphorus were determined in the supernatant solution. The precipitate was dried as described above. The pH 4.7–soluble portion was dried by a vacuum technique and was fractionated with TCA as described for TCA precipitation. Three fractions were obtained: 2% TCA–insoluble, 12% TCA–insoluble, and 12% TCA–soluble.

(b) *TCA precipitation.* Twenty-five per cent TCA solution was added to a solution of the enzyme reaction products until a concentration of 2% was reached; the mixture was then centrifuged, and nitrogen and phosphorus were determined in the supernatant solution. The supernatant was extracted with ether six times to remove the TCA; then the aqueous phase was adjusted to pH 7.0 with NaOH and evaporated to dryness by a rapid vacuum technique (2). The portion of the β-casein insoluble in 2% TCA was dissolved in dilute NaOH and then reprecipitated at pH 4.7 with HCl. The supernatant of the pH 4.7 precipitation was dialyzed against water and dried by the rapid vacuum technique.

Similarly, 50% TCA was added to another portion of the enzyme reaction products until a concentration of 12% was reached. Subsequent steps were the same as those for the 2% TCA precipitation.

Analytical methods. The determination of nitrogen and phosphorus has been described previously (10).

Electrophoresis was carried out with a 1% solution at 1° C. in a Tiselius type electrophoresis apparatus for 3 hr. with a 11-ml. volume, standard, full-length cell. All buffer solutions were of ionic strength 0.1, with 80% contributed by NaCl. The buffering salts were: pH 8.6–veronal, pH 6.7–phosphate, pH 5.6–acetate.

Paper electrophoretic analyses of proteins and peptides were performed in a Durrum type electrophoresis cell (Spinco Model R).[2] Electrophoresis was done at 5° for 16 hr. in a veronal buffer, pH 8.6, ionic strength 0.075, on

[2] It is not implied the USDA recommends the above company or its product to the possible exclusion of others in the same business.

Whatman paper No. 1. Most of the experiments were done with 175 v., which gave a current flow of about 4.5 ma. Usually, 0.01 ml. of a 6% solution of the substance under test was applied to the apex of the paper. Paper chromatography was carried out with n-butanol acetic acid water pyridine as solvent for 16 to 18 hr. by an ascending technique.

Staining was performed with ninhydrin and bromophenol blue (2).

Soluble products. The hydrolysis of β-casein resulting from the action of rennin or pepsin, measured by the amount of soluble nitrogen and phosphorus with three precipitation conditions (pH 4.7, 2% TCA, 12% TCA) is shown in Table 1. Precipitation with 12% TCA gave a very small soluble fraction,

TABLE 1

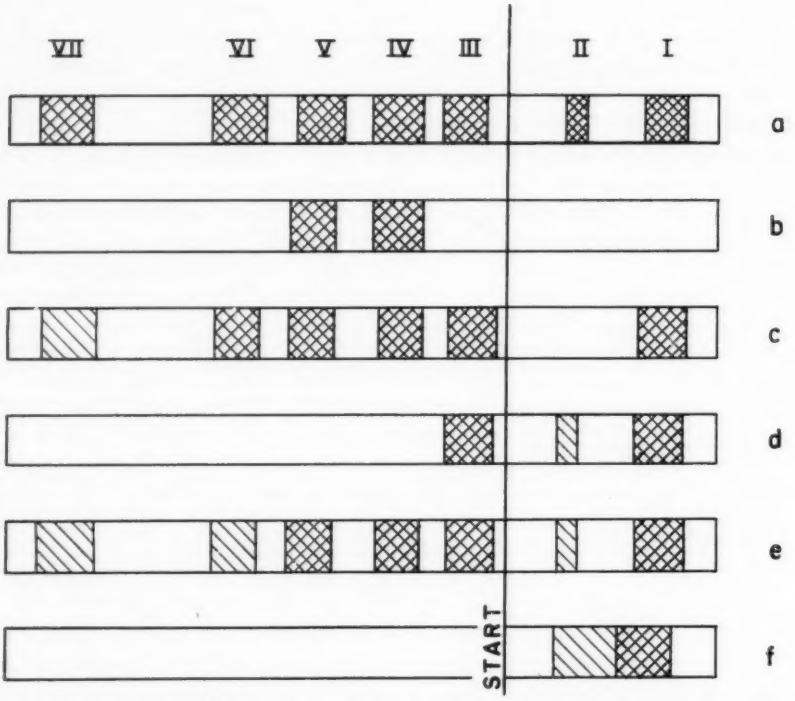
Hydrolysis of β-casein by rennin and pepsin at pH 6.4: soluble nitrogen and phosphorus obtained with three precipitation conditions

Precipitation conditions	Initial soluble	Rennin		Pepsin		
		5 min.	1 hr.	5 min.	1 hr.	5.5 hr.
		Nitrogen (% of total N)				
pH 4.7	0.80	3.46	7.60	3.62	17.49	35.00
2% TCA	0.20	0.92	3.35	1.46	15.81	.....
12% TCA	0.20	0.27	0.98	0.58	2.37	.....
		Phosphorus (% of total P)				
pH 4.7	0.48	2.04	2.39	3.53	2.70	.....
2% TCA	0.15	1.79	1.51	0.77	1.05	.....
12% TCA	0.15	0.85	0.69	0.77	0.77	.....

far less than the amounts obtained by other precipitants. Precipitation at pH 4.7 gave the largest soluble fraction, whereas those experiments with 2% TCA gave somewhat less. Under the conditions used, pepsin hydrolyzed β-casein more extensively than did rennin, particularly with longer periods of time.

The results of paper electrophoretic analysis of pepsin-treated β-casein fractions are shown in Figure 1. The rennin diagram for the pH 4.7–soluble fraction is almost identical with the pepsin diagram shown (a), except that in the Band I region, two very close bands are obtained with rennin. The diagram for the 2% TCA–soluble fraction (not shown) indicates the same number of components as that for the pH 4.7–soluble fraction, but some were reduced in amount. The quantitative relationship between bands shown in Figure 1 was obtained after 5 min. and 1 hr. of proteolysis. The pattern for the pH 4.7–soluble fraction was identical, even when proteolysis was for 5.5 hr. In the pH 4.7–insoluble fraction, however, when proteolysis exceeded 1 hr., the β-casein band was no longer apparent. Bands I and III are the major components of the soluble fractions, with the other cross-hatched bands (Figure 1) intermediate in amount, and the remaining bands relatively minor in amount.

Fractionation of the pH 4.7–soluble material with TCA revealed that the bands corresponding to I, and III to VII, were found in the 2% TCA–insoluble fraction (Figure 1c). All bands in both insoluble fractions gave positive reactions with ninhydrin and bromophenol blue reagents. A qualitative difference between the action of rennin and pepsin was noted in the 12% TCA–insoluble



Schematic representation of paper electrophoretic bands of fractions of β-casein obtained after the action of pepsin for 5 or 60 min. Electrophoresis was at pH 8.6 for 16 hr. (2). Protein bands were evident after staining with bromophenol blue. Cross-hatching is used to indicate the more strongly stained bands. Component IV is β-casein. Components migrating to the left are negatively charged.

FIG. 1. (a) pH 4.7–soluble fraction; (b) pH 4.7–insoluble fraction; (c) pH 4.7–soluble, 2% TCA-insoluble fraction; (d) pH 4.7–soluble, 12% TCA-insoluble fraction; (e) 12% TCA-insoluble, pH 4.7 soluble fraction; (f) 12% TCA-soluble fraction.

fraction (Figure 1d); Band I was the major one for pepsin and Band III was major for rennin. The 12% TCA–soluble fraction was small in amount and contained only one or two bands (Figure 1f). These components gave a positive reaction with ninhydrin only.

β-Casein and all of the insoluble and soluble fractions were studied by paper chromatography. β-Casein and the enzyme-transformed β-casein remained at the origin, with some forward streaking. All other fractions gave spots with greater movement. The results showed that Fractions d and e (See Figure 1) were much more heterogeneous than expected from the electrophoretic results, with movement of 0 to 32 cm. in a 16-hr. run. Further, the major bands (I and III) of e could not be identical with the I and III bands of d, for the number and distribution of the components revealed by chromatography differed greatly

for these two fractions. The 12% TCA–soluble fraction showed six to eight components by chromatography, more than observed by electrophoresis, with movement of 2 to 32 cm. in a 16-hr. run. Differences also were observed between the split products obtained with rennin and pepsin in both the pH 4.7–soluble, 12% TCA–insoluble, and the 12% TCA–soluble fractions.

Insoluble products. The paper electrophoretic pattern of the fraction obtained by precipitation at pH 4.7 is shown in Figure 1b. The 12 and 2% TCA precipitates gave, after reprecipitation at pH 4.7, electrophoretic patterns like Figure 1b. The portion soluble at pH 4.7 obtained by reprecipitation of the 2% TCA–insoluble fraction was heterogeneous as shown by paper electrophoresis (Figure 1c). It contained β-casein (IV), enzyme-transformed β-casein (V), and all the components made soluble by the two enzymes. Both moving boundary and paper electrophoretic studies at pH 8.6 showed that the insoluble fraction was not merely unaltered β-casein, but that a new component differing in electrical charge resulted from the action of rennin and pepsin. This altered casein migrated much faster than β-casein, and slower than α-casein. The concentration of this fraction increased with the reaction time, and in the 5.5 hr. treatment by pepsin all of the original β-casein had been transformed to the new electrophoretic component, which contained 65% of the original β-casein nitrogen (See Table 2). This new electrophoretic fraction was not

TABLE 2

Electrophoretic composition of the insoluble fraction of β-casein (pH 4.7) after hydrolysis with rennin and pepsin

Reaction time with enzymes	pH [a]	Rennin		Pepsin			
		β-casein	Altered β-casein	β-casein	Altered β-casein	Altered β-casein I	Altered β-casein II
		(%)					
5 min.	8.6	92	8	72	28	....	....
1 hr.	8.6	74	26	12	88	....	....
1 hr.	6.7	....	....	11	89	....	....
1 hr.	5.6	....	....	16	84	62	22
5.5 hr.	5.6	....	....	0	100	36	64

[a] pH at which electrophoresis was performed with the moving boundary technique.

homogeneous, for electrophoresis at pH 5.6 revealed two peaks. The ratio of the two peaks was not constant but with time the faster (II) increased at the expense of the slower, as shown in Table 2.

DISCUSSION

The present results comparing the use of several precipitants indicate that the enzymes rennin and pepsin cause a considerable proteolysis of β-casein. This was evident when pH 4.7 or 2% TCA precipitation was used after the action of these enzymes. When 12% TCA was the precipitant, as was the case in the experiments of Nitschmann and collaborators (6, 7), the soluble fraction was negligible. On the basis of similar results, Nitschmann *et al.* (6, 7) had concluded that rennin did not hydrolyze β-casein appreciably, a conclusion that the present results show to be erroneous.

Paper electrophoretic analysis of the soluble fractions showed that the variation in the amount found with the different precipitants was not due merely to difference in degree of solubility of the enzyme-transformed β-casein. There are many more components present in the pH 4.7 soluble fraction than in the 12% TCA–soluble fraction. From this standpoint, the portion soluble at pH 4.7 is the truer measure of hydrolysis.

The amount of proteolysis leading to an increase in the soluble fraction increased with the time of action of the enzymes. The action of pepsin was much more extensive than that of rennin, particularly with longer periods of time. Since the paper electrophoretic patterns of the soluble fraction were essentially the same over a period of 5.5 hr., a general proteolysis probably takes place from the beginning of the reaction. During this same period, free electrophoresis at pH 8.6 shows that the β-casein is transformed to a new electrophoretic component of greater mobility. Electrophoresis at pH 5.6 shows that this fraction is heterogeneous and changes in composition in a regular way with time. Paper electrophoretic patterns and paper chromatography disclosed that the soluble fractions were very complex, containing eight to ten or more components. Some of these components were present only in traces, and it was impossible to give the exact number of peptides.

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NITROGEN FRACTIONS IN CENTRIFUGE PREPARATIONS FROM MILK[1]

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SUMMARY

Concentrations of seven nitrogen fractions were determined in 11 centrifuge preparations from a sample of milk. Larger noncasein nitrogen (NCN) values when the skimmilk entered a rapidly rotating bowl suggest that casein was thus disintegrated or homogenized. Ratios of five NCN fractions to total NCN were found to differ widely, indicating that the composition of whey in a moist sediment differed from that in the corresponding liquid whey. The distribution of NCN fractions differed among the preparations studied.

For many years centrifugation has been almost the exclusive method of separating cream and skimmilk from whole milk. More intense centrifugal fields have been used to separate the casein-containing complex from other proteins in skimmilk (1). This method has been chosen as less likely than precipitation by rennet or acids to modify the kinds or amounts of various proteins (4, 6). A convenient chemical method to determine seven protein fractions in skimmilk recently has been reported (2). Although it was developed for milk, not wheys or sediments, and although each of the fractions is heterogeneous, still it provides evidence of changes in protein distribution.

EXPERIMENTAL PROCEDURE

The per cent nitrogen in each fraction was determined by using the A.O.A.C. macro-Kjeldahl procedure (3) by the analytical laboratory of the Experiment Station. In converting these values to percentage in the original preparation, no correction was applied for the volume of precipitated casein. The 2% correction used for milk (2) would be too small for the sediments and too large for the wheys. Suitable corrections would have been quite uniform within any one group and consistent when comparing groups.

The nitrogen fractions obtained (2) were casein (CN), noncasein (NCN), nonprotein (NPN), β-lactoglobulin (BLN), residual albumin (RAN), proteose-peptone (PPN), and globulin (GN). A drip sample of milk was collected at the receiving tank from a single milking of the Experiment Station herd. Most of this sample was separated, while still warm, in a commercial cream separator to obtain *a*. A small aliquot of the warm whole milk was separated in a Ford bowl (1) to obtain *b*. A third aliquot was stored 48 hr. at 4° C., warmed to 27° C., and then separated as in *b* to obtain *c*.

Aliquots of *a* and a Sharples supercentrifuge were used to prepare four centrifuge wheys and four sediments. Whey *1a* was obtained at room temperature (about 20° C.) by filling the slowly turning bowl with *a*, increasing the

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speed to 40,000 r.p.m. for ½ hr., and allowing the whey to fall from the bowl as rotation ceased. Whey *2a*, also at room temperature, was obtained by bringing the empty bowl to 40,000 r.p.m., filling to overflow, and then pumping in *a* at 15 ml/min until 1 liter whey had overflowed. The whey was collected in a bottle surrounded by ice water. Sediments *3a* and *4a* were obtained by dispersing in water the bottom third and the top third, respectively, of the sediment scraped from the centrifuge bowl after *2a*. The dispersion of these sediments was difficult and incomplete. Undispersed lumps were removed by mild centrifuging in tubes. Preparations *5a* through *8a* were similar, respectively, to *1a* through *4a*, except that the skimmilk *a* and the supercentrifuge were cooled with ice water. The wheys emerged at about 6° C. The amount of water used in reconstituting the four sediments was selected to compromise the expected concentrations of CN and NCN for comparison with skimmilks and wheys.

RESULTS

The results are shown in Table 1. There is some uncertainty in the fourth digit, of the larger numbers, and the fifth digit, where used, serves only to locate the decimal point. The standard errors of duplicate nitrogen determinations, Table 2, ranging from 0.22 to 5.07%, are all small relative to the range of ratios in Paragraph 4 of the Discussion. Table 1 contains two major parts with two subdivisions of each part. Subdivision 1 lists six direct determinations of per cent nitrogen. Four of these are not included among the seven fractions listed above, but were used to obtain by difference the remaining five fractions of Subdivision 2. Part 2 consists of ratios. Subdivision 3 (lower right) lists ratios of CN, NPN, BLN, RAN, PPN, and GN to NCN. Subdivision 4 (lower left) contains ratios of these ratios for the two pairs of wheys and the two pairs of sediments.

DISCUSSION

The lower value of per cent CN in *c* than in *a* or *b* may be partly due to greater clustering or aggregation of the previously cooled fat and casein. The corresponding decrease in volume (per cent CN × 6.38 × 0.7) would explain only about 2% of the progressive increase (*a b c*) in NCN. The larger NCN in *2a* than in *1a*, *6a* than in *5a*, *4a* than in *3a*, *7a* than in *8a* suggests that some of the casein complex has been disintegrated or homogenized when entering the rapidly rotating Sharples bowl, so that it is no longer precipitated by trichloracetic acid. That CN for *5a* is larger than for *6a*, whereas *2a* is larger than *1a*, may also be partly due to greater disintegration of the more voluminous cold micelles (7). This greater voluminosity when cold would also explain the greater increase of NCN *6a-5a* than *2a-1a*. The largest of these CN values is small enough that differences in CN are here also not enough to explain, on the basis of the volume not occupied by casein, the differences in NCN. The larger values for CN in *5a* than in *1a*, and in *6a* than in *2a*, are due to the slower and less complete sedimentation when cold.

The higher values of per cent NCN for tops, *4a* and *8a*, than for bottoms, *3a* and *7a*, are probably partly due to larger amounts of the wheys in the moist

TABLE 1

Distribution of nitrogen fractions in centrifuge preparations from a single milk sample

Preparations		Skims			Wheys				Sediments			
		a	b	c	1a	2a	5a	6a	3a	4a	7a	8a
Fractions		10,000 × % *N* in Fraction										
1. Direct												
TN	I	5,798	5,879	5,651	1,900	2,046	2,248	2,244	10,530	11,230	9,895	9,803
NCN − PPN	II	1,267	1,305	1,379	1,549	1,594	1,314	1,579	377	381	239	270
TAN + NPN	III	1,164	1,175	1,275	1,314	1,434	1,246	1,372	214	239	217	189
BLN + NPN	IV	838	856	845	951	1,031	894	964	159	158	117	134
HAc filtrate	NCN	1,375	1,424	1,460	1,625	1,671	1,377	1,683	621	677	355	399
Cl_3 HAc filtrate	NPN	300	325	111	392	401	367	395	111	83	80	86
2. Difference												
I—NCN	CN	4,423	4,455	4,191	275	375	871	561	9,909	10,550	9,540	9,404
IV—NPN	BLN	538	531	734	559	630	527	569	48	75	37	48
III—NPN—BLN	RAN	326	319	430	363	403	352	408	55	81	100	55
NCN—II	PPN	108	119	81	76	77	63	104	244	296	116	129
NCN—III—PPN	GN	103	130	104	235	160	68	207	163	142	22	81
4. 100 × ratios of ratios of −*a*.					3. 1,000 × ratios to NCN							
1a/ 5a/ 3a/ 7a/ 2a 6a 4a 8a												
100 114 146 104	CN	3,217	3,129	2,871	169	224	633	333	15,950	15,590	26,870	23,570
91 113 69 87	NPN	218	228	76	241	240	267	235	179	123	225	216
93 106 74 204	BLN	391	373	503	344	377	383	338	77	111	104	120
102 74 90 101	RAN	237	224	295	223	241	256	242	89	120	282	138
151 40 125 31	PPN	79	84	55	47	46	46	62	393	437	327	323
	GN	75	91	71	145	96	49	123	262	210	62	203

TABLE 2

Standard deviations of nitrogen determinations. Per cent of averages of duplicates

N fraction	I	II	III	IV	V	VI
Skims	2.52	2.84[a]	1.38	1.12	1.36[a]	0.25
Bottom sediments	2.45	5.07	1.20	3.99	1.87	1.43
Top sediments	3.48	1.95	1.61	0.22	2.70	2.25
All	2.55	3.50	1.42	2.74	2.72	1.64

[a] Deviations of two preparations averaged, not standardized.

tops. If this were the only cause, the variations in the five fractions making up the NCN should be proportional. Large differences in these ratios, among wheys, among sediments, and among skimmilks are found in Subdivision 3. The composition of an adsorbed whey must then differ from the corresponding free liquid whey or else whey proteins must be selectively adsorbed in the dry part of the moist sediment. Identical ratios of CN, serum nitrogen, and organic phosphorus to TN in sediments washed or not washed with water (5) make the latter explanation less likely.

In Subdivision 4 the range of ratios of NCN fractions to NCN (highest/lowest) is much greater for CN (151/31 = 4.9) and RAN (204/74 = 2.7) than for BLN (113/69 = 1.6), NPN (146/100 = 1.5), or PPN (102/74 = 1.4). These ranges of ratios are very effective evidence that protein distribution differed among the preparations studied.

Most statements regarding the precision and homogeneity of separations were descriptive. Numerical comparisons with other methods involved different samples and different investigators (2).

The separations reported here followed several partial preliminary separations used to become familiar with the method, and to adjust quantities of various fractions and amounts of Kjeldahl reagents. These preliminary tests confirm the finding from Table 1 that the different methods of centrifuging produce different distributions of nitrogen fractions.

No attempt has yet been made to select best or critical temperatures and separator speeds. It is doubtful if differences or ratios produced by an attempted repetition of the experiment would exactly duplicate the values in Table 1. It seemed unwise to delay reporting differences until their nature and precision are more completely determined.

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EFFECT OF THE STRUCTURE OF SOME MERCAPTANS ON THEIR AMPEROMETRIC TITRATION WITH SILVER NITRATE[1]

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SUMMARY

The mercaptans studied were L-cysteine hydrochloride, DL-homocysteine thiolactone hydrochloride, S-methyl-L-cysteine, thioglycolic amide, N-acetyl-DL-penicillamine, N-acetyl-DL-homocysteine thiolactone, L-cysteine methyl ester hydrochloride, 2-mercaptoethylamine hydrochloride, DL-penicillamine hydrochloride, α-toluenethiol, and sodium thioglycolate. These mercaptans yielded theoretical titrations with silver nitrate, except cysteine hydrochloride and penicillamine, which were about 25% too high in titer. Cysteine has three ionized forms whereby the carboxyl group is completely ionized at the pH of the amperometric titration, while at pH 11 or higher, the $-SRNH_2$ form predominates, but at pH 9.0 it is negligible and the $-SRNH^+_3$ form predominates. Ultra-violet absorption analyses indicated that silver ions react not only with the −SH groups, but at some other site in the molecule, probably involving the ionic forms, $-SRNH^+_3$, $-SRNH_2$, and complex formation.

The amperometric titration with the rotating platinum electrode was first described by Laitinen and Kolthoff (4). It has proven useful in estimating sulfhydryl (SH) groups which are the most reactive ones in proteins. They oxidize to the stable, covalent, cross-linked, disulfide bond. The method of Kolthoff's (2) was used to determine −SH groups in milk heated to different temperatures. When the titrating apparatus was standardized, it was found that the silver nitrate titer for cysteine hydrochloride was always about 25% too high. The literature revealed similar results (5, 6). The reasons for these discrepancies were studied.

Excellent reviews of the literature on amperometric titrations have been published by Laitinen (3).

EXPERIMENTAL PROCEDURES

The amperometric titrating apparatus consisted of a rotating platinum electrode, a calomel electrode, a galvanometer,[2] burette, and nitrogen which was deoxygenated in alkaline pyrogallol. A Cary recording spectrophotometer was used for the ultra-violet absorption analyses.

The mercaptans studied were L-cysteine hydrochloride, DL-homocysteine thiolactone hydrochloride, S-methyl-L-cysteine, and thioglycolic amide, N-acetyl-DL-penicillamine, N-acetyl-DL-homocysteine thiolactone, L-cysteine methyl ester hydrochloride, 2-mercaptoethylamine hydrochloride, DL-penicillamine hydrochloride, α-toluenethiol, and sodium thioglycolate. These mercaptans were diluted to 0.001 *M* with distilled water, except homocysteine thiolactone and N-acetyl-DL-homocysteine thiolactone, which were dissolved in ammonium hydroxide

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[2] Made by the Wilkens-Anderson Company, Chicago, Illinois.

to break the lactone ring and then they were diluted to 0.001 *M* with distilled water.

A buffer of 0.1 *M* sodium acetate and ammonium hydroxide was used for the amperometric titrations and spectrophotometric analyses.

The amperometric titrations were made by adding 90 ml. of 0.1 *M* sodium acetate and 3 ml. of ammonium hydroxide (sp. gr. 0.9) to a 250-ml. beaker. Oxygen-free nitrogen gas was passed into this solution through a sintered glass bubbler. Ten milliliters of 0.001 *M* mercaptan solution was added to the reaction beaker. When the platinum electrode was placed in the reaction beaker, the galvanometer reading was 20 to 30 microamperes, which decreased to near zero within 5 to 10 min. after adding the sample. The galvanometer reading was adjusted to zero. The mercaptans were titrated with 0.001 *M* silver nitrate. The diffusion current was recorded after each increment of silver nitrate. A plot of current versus milliliters of silver nitrate showed a sharp break at the end point. After each titration the platinum electrode was wiped with soft tissue paper and cleaned with concentrated nitric acid and distilled water.

RESULTS AND DISCUSSION

Eleven different mercaptans were titrated, amperometrically, with silver nitrate, and the curves are presented in Figures 1 and 2. All of them yielded theoretical or close to theoretical values, except cysteine hydrochloride and penicillamine, which were almost 25% too high in titer. Homocysteine thiolactone yielded the theoretical value and it has almost the same structure as cysteine and penicillamine, except it has a longer chain. It is necessary to break the lactone ring of homocysteine thiolactone in order to obtain a theoretical

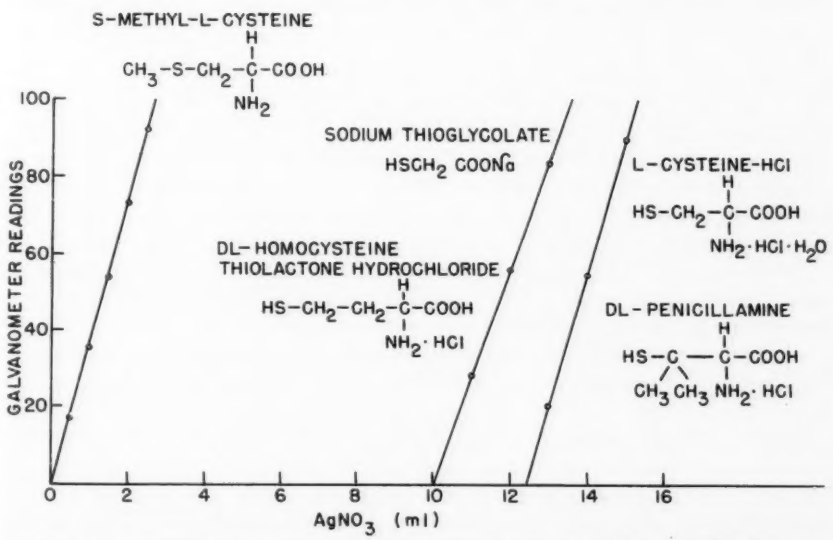

FIG. 1. Amperometric silver titration of mercaptans with different structures.

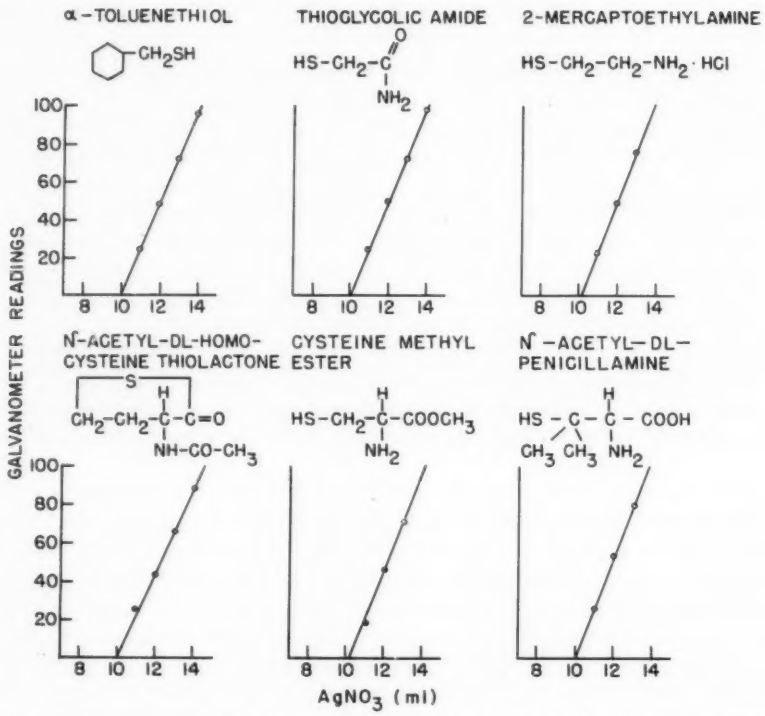

FIG. 2. Amperometric silver titration of mercaptans that yielded theoretical results.

titration value. If the lactone ring is not broken the titration will be too low. Other mercaptans which yielded theoretical values have −SH group only (α-toluenethiol) or a combination of −SH and −COOH groups (sodium thioglycolate, N-acetyl-DL-homocysteine thiolactone, which was prepared in the same manner as homocysteine thiolactone and N-acetyl-DL-penicillamine). The others which have −SH and $-NH_2$ groups (2-mercaptoethylamine, cysteine methyl ester, and thioglycolic amide) yielded close to theoretical values. S-methyl-L-cysteine does not form the silver mercaptide because of the methyl group attached to the sulfur atom. After cysteine reacted with N-acetyl malemide or formaldehyde, the amperometric titrations were zero.

Aspartic acid and serine have structures similar to cysteine and have about the same pK constants for the amino and carboxyl groups, but they do not have −SH groups (Table 1). These two amino acids were titrated, amperometrically. Their amino and carboxyl groups did not react with silver nitrate.

The amperometric titrations for cysteine at pH 8.5, 9.8, and 11.2 were 50, 30, and 25%, respectively, above the theoretical value. Benesch *et al.* (1) showed that cysteine has three ionized forms. The carboxyl group is completely ionized at the pH of the amperometric titration and at pH 11 and higher the

TABLE 1

Dissociation constants for aspartic acid, cysteine hydrochloride, and serine

Amino Acid	Dissociation constants		
	pK_1	pK_2	pK_3
Aspartic acid ($HOOC\text{-}CH_2CHNH_2$ COOH)	1.88[a](COOH)	3.65[a](COOH)	9.60[a](NH^+_3)
Cysteine hydrochloride ($HS\text{-}CH_2CHNH_2HCl$ COOH)	1.96[b](COOH)	8.18[b](NH^+_3)	10.28[b](−SH)
Serine ($HO\text{-}CH_2CHNH_2$ COOH)	2.21[c](COOH)	9.15[c]	

[a, c] 25° C.
[b] 30° C.

$-SRNH_2$ form predominates, but below pH 9.0 it is negligible and the $-SRNH^+_3$ form predominates. It appears that the higher amperometric titration value at the lower pH is concerned with ionic form, $-SRNH^+_3$ in cysteine. Nevertheless, at a pH above 11, the value was still 25% above theoretical. This suggests that the silver ions react in ways other than with the −SH groups in cysteine. Representative results are shown in Figure 3. Effect of temperature on the reaction of silver ions with cysteine is shown in Figure 4. At 4° C. the titration value was 35% higher than theoretical, but at 60 to 70° C. it was nearly theoretical. The higher temperatures did not oxidize the −SH groups, because the cysteine solution had the same silver nitrate titer after it was cooled as it had before it was heated.

The reaction at the end point. To study further the reaction at the end point in the amperometric titration of cysteine, the sensitivity of the galvanometer was increased three times. Immediately after each addition of silver nitrate, the galvanometer was read at half-minute intervals for 3 min. After adding 10, 11, and 12 ml. of silver nitrate, the galvanometer reading gradually decreased to zero (Figure 5). This means that ten molar equivalents of cysteine

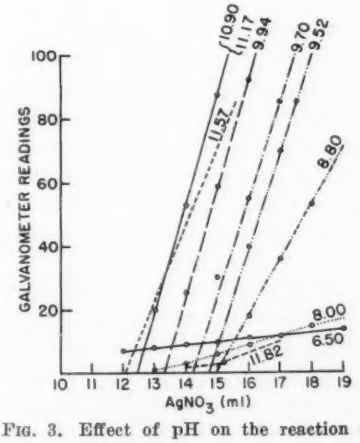

FIG. 3. Effect of pH on the reaction of silver ions with cysteine.

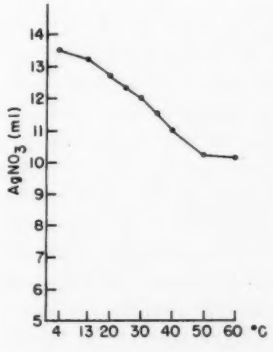

FIG. 4. Effect of temperature on the reaction of silver ions with cysteine.

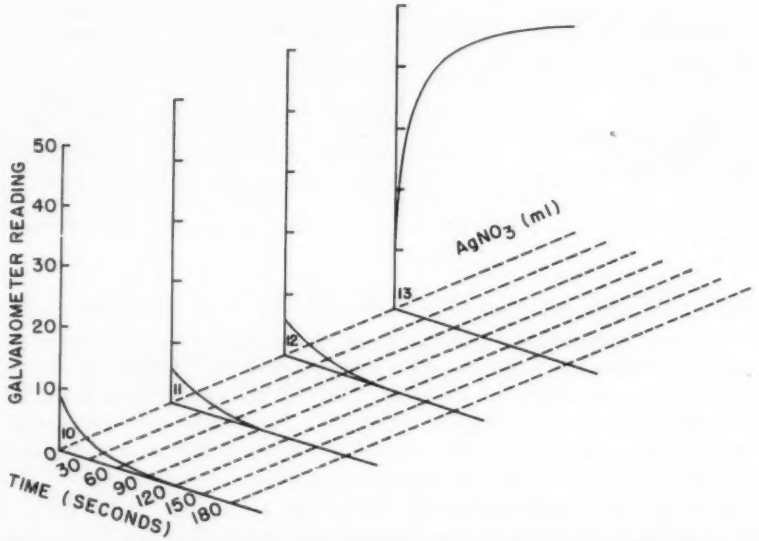

FIG. 5. The behavior of the amperometric silver titration near the end point.

combined with more than ten molar equivalents of silver ions. After adding 13 ml. of silver nitrate, the galvanometer reading gradually increased to a stable value. Therefore, the removal of silver ions from solution is slow between the theoretical end point and the point where they are in excess.

Back titration. The results of the back titration, in which 10 ml. of 0.001 *M* cysteine hydrochloride were titrated against 10 ml. of 0.001 *M* $AgNO_3$, are shown in Figure 6. The end point of the back titration curve was 8 ml. of cysteine which combined with ten molar equivalents of silver ions and there were no free silver ions in the solution. After 10 ml. of cysteine had been titrated against 10 ml. of $AgNO_3$, the other portion of silver nitrate was titrated with cysteine hydrochloride continuously. If all $-SH$ groups reacted with silver ions and cysteine could not react with additional silver ions in its molecular or silver-cysteine complexes, then the immediate increase of current must be due to free silver ions. However, the titration curve for silver nitrate was the same as the one for cysteine. The results show that cysteine reacted with silver ions at the $-SH$ position to form silver mercaptide and it probably bound silver ions to form complexes.

Ultra-violet absorption analyses. Since it is probable that the ionic forms, $-SRNH^+_3$ and $-SRNH_2$, react with silver ions to form complexes in the amperometric titration of cysteine, it was decided to determine the ultra-violet absorption spectra for cysteine at different pH values and also for the silver-cysteine complexes. The mercaptan solutions were prepared in the same way as they were for the amperometric titrations. At the lower pH, the maximum absorption for cysteine was at a shorter wave length than it was at the higher

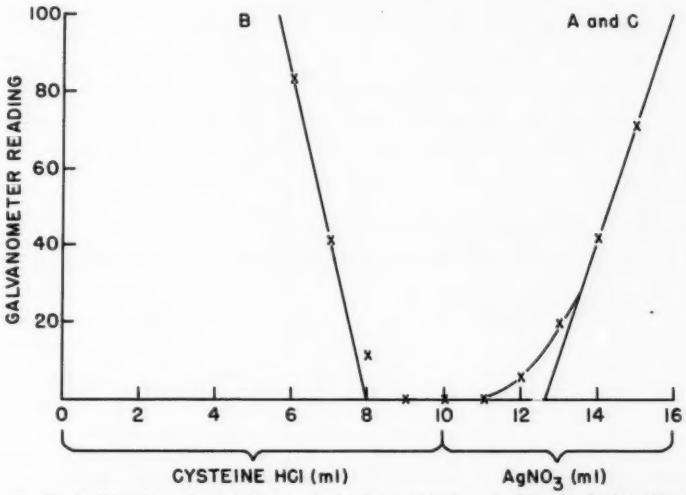

FIG. 6. Back titration of cysteine against silver nitrate. A. Standard titration, B. Back titration, C. Titration after back titration.

pH, being 238 mμ at pH 11.2. The results are shown in Figure 7. Silver nitrate, 0.001 *M*, was added to equal molar volumes of cysteine hydrochloride at different pH values. The ultra-violet absorption data are shown in Figure 8. Regardless of pH, the maxima absorption of the cysteine-silver complexes were all the same at 270 mμ.

From 1 to 20 ml. of 0.001 *M* $AgNO_3$ was added to 10 ml. of 0.001 *M* cysteine solution. The pH of the reacting solutions was 11.1. Representative results

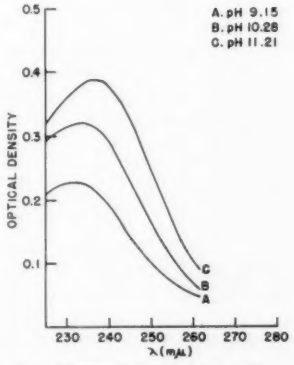

FIG. 7. Absorption spectra of cysteine at different pH values.

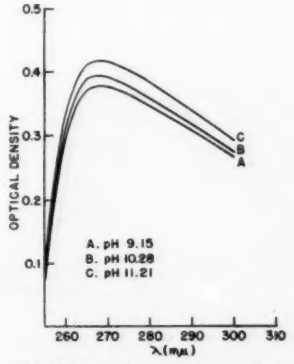

FIG. 8. Absorption spectra of cysteine-silver complexes at different pH values with initial concentration of cysteine at 0.001 and silver nitrate 0.001 *M*.

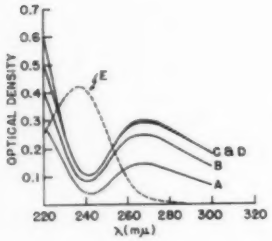

FIG. 9. Effect of the concentration of silver nitrate on cysteine-silver complexes. A. 10 ml. of cysteine and 7 ml. of $AgNO_3$, B. 10 ml. of cysteine and 10 ml. of $AgNO_3$, C. 10 ml. of cysteine and 13 ml. of $AgNO_3$, D. 10 ml. of cysteine and 20 ml. of $AgNO_3$, E. Cysteine hydrochloride.

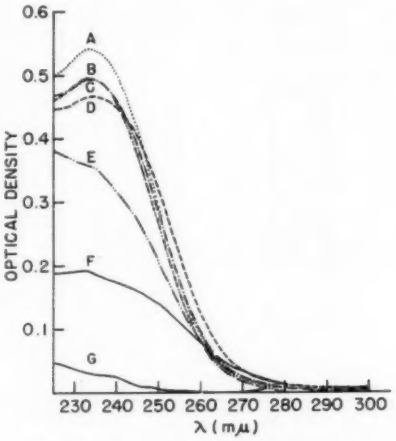

FIG. 10. Absorption spectra of some mercaptans with different structures in 0.0001 *M* concentration.
A. Cysteine methyl ester HCl, B. Cysteine HCl, C. 2-mercaptoethylamine HCl, D. DL-penicillamine, E. N-acetyl-DL-penicillamine, F. Sodium thioglycolate, G. S-methyl cysteine.

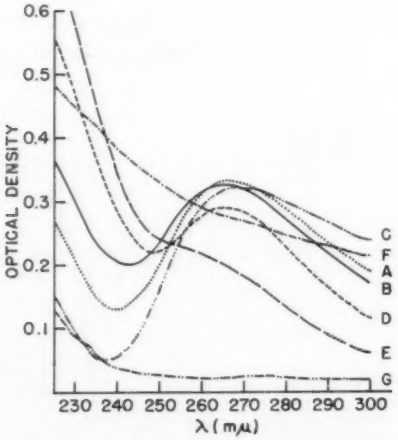

FIG. 11. Absorption spectra of silver complexes.
A. Cysteine methyl ester HCl, B. Cysteine HCl, C. 2-mercaptoethylamine HCl, D. DL-penicillamine, E. N-acetyl-DL-penicillamine, F. Sodium thioglycolate, G. S-methyl cysteine.

are shown in Figure 9. The maximum absorption of silver was below 220 mμ and for cysteine it was 238 mμ. Maximum absorption at 270 mμ increased with the addition of silver ions; however, the maximum peak at 270 mμ was obtained with 13 ml., and 20 ml. of $AgNO_3$ changed the peak only slightly. This verifies the results of the amperometric titration whereby 10 ml. of 0.001 *M* cysteine required 13 ml. of 0.001 *M* $AgNO_3$ to cause a rapid increase in the diffusion current at the end point. This indicates that the silver ions react not only with the −SH group but also at some other site in the molecule.

Absorption spectra of cysteine, penicillamine, cysteine methyl ester, N-acetyl penicillamine, sodium thioglycolate, 2-mercaptoethylamine, and s-methyl cysteine are shown in Figure 10. The absorption spectra of the silver complexes of these mercaptans are shown in Figure 11. It is evident that both sodium thioglycolate and N-acetyl penicillamine, which have COOH and −SH groups, do not show the characteristic absorption curve at 270 mμ like the other mercaptans, which have −COOH, $-NH_2$, and −SH groups or −SH and $-NH_2$ groups.

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IDENTIFICATION OF SOME VOLATILE CARBONYL COMPOUNDS FROM NONFAT DRY MILK [1]

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SUMMARY

Evidence is presented, in the form of compound identification, that lipid oxidation, as well as Maillard-type browning, occurred in instant nonfat dry milk (NFDM) stored at room temperature for 1 yr. The compounds identified from instant NFDM were formaldehyde, acetaldehyde, acetone, butanone, methylpropanal, 3-methylbutanal, furfural, diacetyl, hexanal, and nonanal. Evidence is also presented for the presence of heptanal, octanal, decanal, dodecanal, and tetradecanal. The isolated compounds are believed to be largely responsible for the cereal-type flavor which sometimes develops in NFDM. It was estimated that the flavorful carbonyl compounds existed in reconstituted NFDM at levels of a few parts per billion.

The recent development of instant forms of nonfat dry milk has greatly increased the utility of this product for reconstituted beverage purposes (1). This trend to increased beverage use naturally places a higher premium on the flavor quality. The main flavor problem encountered in NFDM appears to be the development of a cereal-like flavor. This flavor characteristic may be likened to the well known flavors of toasted breakfast cereals prepared from wheat or corn. The purpose of this study was to identify compounds responsible for cereal flavor in NFDM, to help define the chemical mechanisms of this type of flavor development.

EXPERIMENTAL PROCEDURE

Preparation of sample. Commercial samples of instant NFDM powder as well as its precursor, noninstant powder, were packaged in 50- and 100-lb. bags, each with two thicknesses of polyethylene in addition to two thicknesses of fiber, and were stored at room temperature for a year. Comparison of the flavor of these milks, when reconstituted, indicated that the instant milk powders possessed a stronger cereal-like flavor than did the noninstant powders. The instant variety of NFDM was used in an attempt to isolate and identify chemical compounds associated with the off-flavors.

Observations on volatiles recovered from the stored milk powder by low-temperature, reduced-pressure distillation. Preliminary studies with a low-temperature, reduced-pressure distillation technique, similar to that employed

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by Day *et al.* (3), were conducted. The method employed differed from the Day method in that an additional ethanol dry ice trap was used in place of liquid nitrogen traps, and a water-cooled condenser was placed after the first receiver in a reflux position to facilitate collection of most of the water in the first receiver. Twelve hundred grams of instant NFDM powder, reconstituted into a final volume of six liters and containing 1 ml. of G. E. Antifoam-60, was distilled for 2 hr. to yield two liters of distillate in the first trap.

The odor of the various traps was characterized as malty, cereal-like, and oily. Portions of the combined material from the traps imparted a malty, cereal-like flavor when added to fresh milk. The odor of the pot residue was less intense but still typical of the NFDM. Qualitative chemical tests on the distillate revealed that practically all of the odor was destroyed by aqueous acidic 2,4-dinitrophenylhydrazine. The odor remaining resembled that of methyl sulfide (11). These observations indicated that while the distillation procedure was not efficient, the odorous material recovered was typical of the flavor associated with the NFDM. Furthermore, the bulk of the odorous compounds appeared to be carbonyls.

Accumulation of identifiable quantities of 2,4-dinitrophenylhydrazones (DNPH's) by modified distillation techniques. Once the flavorful compounds of the distillate were characterized as carbonyl, the distillation procedure was modified. Aqueous acidic 2,4-dinitrophenylhydrazine solutions were employed in place of the ethanol dry ice traps and the distillate was entrained directly into the reagent. The first two-liter receiver was used, as before, to collect the bulk of the water from the distillation. The first reagent trap had a capacity of one liter and was charged with 250 ml. of 3 *N* H_2SO_4 solution containing 100 mg. of 2,4-dinitrophenylhydrazine. The following two 500-ml. traps each contained 50 mg. of 2,4-dinitrophenylhydrazine in 50 ml. of 2 *N* H_2SO_4. The distillation procedure was otherwise identical to that previously described. By using this method, 72 kg. of instant nonfat dry milk powder was distilled in eight-liter reconstituted batches over a period of several weeks. The combined DNPH solutions from a day's operation (usually three distillations) were adjusted to approximately 1 *N* with H_2SO_4 and extracted (within 2-24 hr. of the distillation) four times with 200-ml. volumes of petroleum ether.

In addition to the petroleum ether extractable DNPH's, a hydrazone with unusual solubility properties was isolated. This hydrazone was thrown out of the reaction mixture during the routine extractions with petroleum ether. Study of this hydrazone indicated that the parent compound was diacetyl (2,3-butanedione). This was based upon the following evidence: treatment of the derivative with alcoholic alkali yielded a blue color, indicating a dicarbonyl; the UV absorption spectrum of the unknown DNPH was identical to diacetyl-bis-DNPH; and an odor typical of diacetyl was observed when the derivative was dissolved in concentrated H_2SO_4 followed by dilution with two volumes of water.

The combined derivatives extracted into petroleum ether were concentrated by evaporation and fractionated by nitromethane-hexane partition chromatography (2). The step-wise separation of these hydrazones was performed by the same procedure previously described for the chromatography of cheese

hydrazones (2). The chromatographic threshold volumes obtained from this procedure were also useful for identification purposes. Once separation was accomplished on the chromatographic columns, eluates of the individual bands were evaporated to dryness under reduced pressure, and when possible, the dry residues were recrystallized from petroleum ether. A number of identification techniques, such as melting points, mixture melting points, absorption spectra, color reactions with alcoholic alkali, and odors upon regeneration (7) were applied as appropriate to the isolated fractions. The separated DNPH'S and the criteria employed for their identification are presented in Table 1.

Estimation of quantities of carbonyl compounds in NFDM. Two different approaches were used to gain information on the approximate concentrations of the identified (Table 1) carbonyl compounds in the NFDM and to ascertain if they had flavor significance. The quantities of carbonyl compounds isolated from NFDM and raw skimmilk (control) were estimated by spectrophotometric measurement of the isolated DNPH's. Synthetic blends of the identified compounds were then added to fresh skimmilk and judged for flavor.

The same vacuum distillation procedure used for isolation of the carbonyl compounds in Table 1 was used to distill three eight-liter batches of the reconstituted (9%) instant NFDM. After the completion of each distillation, the solutions in the reagent traps were added to the two liters of distillate in the first ice-cooled trap. The acid strength of the mixture was adjusted to 1 *N* H_2SO_4 and it was then allowed to stand at room temperature for 6 hr. The reaction mixture was extracted three times with $CHCl_3$ to yield 400 ml. of extract, which was then washed three times with 100-ml. portions of water. The washed $CHCl_3$ extract from the three distillations was then combined and evaporated to dryness under reduced pressure. The dry deposit of hydrazones and excess reagent was extracted with successive 20-ml. portions of equilibrated hexane until there was no evidence of hydrazones in the last extract. Each 20-ml. extract was chromatographed on a separate 20-g. Method B column (2) and the extraction was stopped when it was evident, by the appearance of the developed columns, that all of the hydrazones had been extracted. The foreruns from the B columns, containing hydrazones of C_5 and higher carbonyls, were combined and evaporated to dryness under reduced pressure. The residue, dissolved in 10 ml. of hexane, was chromatographed on a 60-g. Method A column (2). Appropriate eluate fractions from both the A and B columns were collected, evaporated to dryness, and the residues dissolved in methanol for subsequent spectrophotometric observations.

The DNPH mixture obtained from the distillate of eight liters of raw skimmilk (control) contained only the short-chain carbonyls. This mixture was resolved by using Column B (2). The approximate concentration of each carbonyl, isolated from the NFDM and raw skimmilk control samples, was determined by measuring absorbance at the wavelength of maximum light absorption of each purified DNPH dissolved in a known volume of methanol (2). A molar absorptivity of 23,000 was used for all DNPH's of monocarbonyls, except furfural, for which 27,000 was used (6). The quantitative data pre-

TABLE 1

Summary of identification data on 2,4-dinitrophenylhydrazones from reconstituted nonfat dry milk

2,4-DNPH	Chromatographic threshold vol. *(ml.)*		Corrected melting points (° C.)			λ Maximum, mμ [a]		Odor comparison of regenerated carbonyl with suspected carbonyl	Comments
	Unknown	Authentic	Unknown	Authentic	Mixture	Unknown	Authentic		
Column Method A									
2-Butanone	22.6	19.0	115	117	.....	363	363	.....	
Methylpropanal	24.0	25.0	183	182	182	358	358	.....	
3-Methylbutanal	18.5	19.5	122	123	122	358	358	.....	
Hexanal	15.9	15.4	104	104	105	358	358	Identical	
Heptanal	12.5	12.2	.....	.....	.....	360	358	Identical	
Octanal	9.6	10.2	.....	.....	.....	358	358	Identical	
Nonanal	8.0	8.2	106	106	106	358	358	Identical	
Decanal	6.5	6.9	.....	.....	.....	358	358	Identical	
Dodecanal	5.1	5.1	.....	.....	.....	358	358	Identical	
Tetradecanal	4.0	.....	.....	.....	.....	358	358	Same as dodecanal	
Column Method B									
Formaldehyde	70.0	70.9	163	166	165	346	346	.....	Orange, alc. alk.
Acetaldehyde	45.0	47.5	165	168	165	356	356	.....	
Acetone	29.2	30.3	125	126	123	.....	363	.....	
Furfural									
(cis)	49.8	44.5	.....	.....	.....	376	379	.....	Pink, alc. alk.
(trans)	124.0	123.0	.....	.....	.....	385	386	.....	Pink, alc. alk.
Diacetyl	.....	.....	.....	.....	....	394	394	Identical	Blue, alc. alk.

[a] Determined in methanol.

sented in Table 2 are expressed in micrograms of free carbonyl per liter of milk. It is noted that decanal and tetradecanal were not detected in this experiment, nor were quantitative data obtained for the dicarbonyl compounds. Allowances were also made in the data for the quantities of formaldehyde, acetaldehyde, and acetone found in the chloroform used for extractions (2).

TABLE 2

Quantities of carbonyl compounds isolated from reconstituted (9% solids) nonfat dry milk and raw skimmilk

Suspected compound	Evidence of identification as 2,4-dinitrophenylhydrazones				Approximate quantities of free carbonyls isolated per liter of milk *(micrograms)*
	Chromatographic threshold vol. *(ml.)*		λ Maximum, mμ		
	Unknown	Authentic	Unknown	Authentic	
Column Method A					
NFDM					
Methylpropanal	22.7	25.0	358	358	0.33
3-Methylbutanal	19.6	19.5	358	358	0.17
Hexanal	15.0	15.4	358	358	0.38
Heptanal	12.5	12.2	359	358	0.23
Octanal	10.2	10.2	362	358	0.17
Nonanal	7.5	8.2	358	358	0.46
Dodecanal	5.2	5.1	358	358	2.82
Column Method B					
NFDM					
Formaldehyde	62.6	70.9	346	346	1.92
Acetaldehyde	41.4	47.5	355	356	11.90
Acetone	26.5	30.3	363	363	7.20
Furfural					
(cis)	49.8	44.5	376	379	2.38
(trans)	124.0	123.0	385	386	1.20
Butanone	16.8	18.5	363	363	0.21
Raw skimmilk					
Formaldehyde	70.0	70.9	346	346	1.90
Acetaldehyde	41.0	47.5	354	356	0.45
Acetone	28.5	30.2	362	363	114.00

Organoleptic properties of synthetic carbonyl compounds added to fresh skimmilk. Accurately weighed quantities of a few milligrams of each of the carbonyl compounds, for which analytical data are presented in Table 2, were added to fresh pasteurized skimmilk while mixing the milk in a Waring Blendor. This stock mixture was diluted to yield three different concentration levels of the compounds in the same proportions as listed in Table 2. These levels were: (a) same as level in Table 2; (b) ten times this level; (c) 30 times this level. These three samples, together with the control skimmilk and a sample of reconstituted NFDM, were evaluated for flavor by three experienced judges. The results are presented in Table 3.

DISCUSSION

Efficiency of distillation was sacrificed in this experiment in order to provide mild treatment of the milk. This minimized chances of decomposition or

alteration of compounds during isolation. Although recovery studies of the carbonyls were not conducted on skimmilk, previous work with the distillation apparatus indicated that the quantities of compounds listed in Table 2 represented less than 10% of the amounts actually present in the milk (2). Organoleptic observations indicated this assumption to be sound, since the addition of ten times the quantities of isolated compounds (Table 3) to fresh skim-

TABLE 3

Organoleptic evaluation of milks

Judge	Control, fresh pasteurized skimmillk	Control plus quantities of carbonyl compounds isolated from NFDM (Table 2)	Control plus 10 times quantities in Table 2	Control plus 30 times quantities in Table 2	Reconstituted instant NFDM [a]
1	Cooked	Cooked	Cereal	Tallowy	Cereal
2	Cooked	Cooked	Cereal	Tallowy	Malty
3	Cooked	Cooked	Malty	Cereal	Cereal

[a] Sample of the same NFDM from which the identified compounds were isolated. This sample had been stored in a polyethylene bag for 18 mo. at room temperature.

milk resulted in the typical cereal-like flavor. The lower concentration (Table 3) was not detectable, whereas concentration of 30 times that found in NFDM was characterized as tallowy.

The nature of the compounds identified in this study indicate that both Maillard-type browning and lipid oxidation had occurred in the instant NFDM. Diacetyl and furfural are classical products of sugar fragmentation and dehydration during the browning reaction (5). The branched-chained aldehydes, 3-methylbutanal and methylpropanal, may be formed by the Strecker degradation of the amino acids, leucine and valine, respectively. This is another well known pathway of the browning reaction (5). The C_6-C_{14} aldehydes presumably resulted from oxidation of lipid material in the dry milk. So-called nonfat dry milk contains approximately 0.5% fat. Saturated aldehydes are characteristic products of lipid oxidation (10). The simulation of the cereal-like flavor by the addition of the identified compounds to fresh skimmilk indicates that lactose-protein browning reactions and lipid oxidation reactions play significant roles in the formation of flavor compounds. The evidence implicating browning reactions in flavor production in NFDM confirms previous suggestions to this effect (4, 8, 9). The identification of the flavorful C_6-C_{14} aldehydes in NFDM is direct evidence that lipid oxidation may also be involved in flavor production. Henry *et al.* (4), in a comprehensive study of the effect of various storage conditions on the production of flavor defects in NFDM, observed flavor production in samples of low-moisture content, in which no significant browning had taken place, and suggested that the flavor was produced by lipid oxidation.

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SEDIMENTATION OF RECONSTITUTED CONCENTRATED MILKS[1]

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SUMMARY

Heating milks above sterilization temperatures resulted in increased rates of sedimentation during ultracentrifugation and packing of particles after sedimentation. Sedimentation constants for raw milks at time of maximum depth of sediment were about 400 $\times$ 10^{-13} cm.$^{-1}$ sec.$^{-1}$. For sterile milks the constants ranged from 900 to 3,000 $\times$ 10^{-13}. When milks were concentrated, either in a commercial vacuum pan or in a glass vacuum flask, including milks concentrated under aseptic conditions, the sedimentation curves of the reconstituted milks were similar to those of the same milks before concentration. Reconstituted sterilized concentrated milks yielded sedimentation constants from 15,000 to 114,000 $\times$ 10^{-13}. Substituting estimated values for the density of the particles in the equation, $d = \sqrt{\frac{18\,\eta s}{\rho - \rho_o}}$, it was found that they should be microscopically visible at a magnification of 450. When microscopically estimated measures of the diameters were substituted in the equation, the value of the expression, ρ-ρ_o, was about 0.1. Microscopic examination, with a phase microscope, revealed that the larger particles in sterile concentrated milks were made up of nuclei or irregular particles surrounded by protein material. In many reconstituted milks all of the fat sedimented with the protein.

Ultracentrifugation studies have shown that the casein particles in normal untreated milks have a range of sizes, with sedimentation constants of 180 to 4,950 $\times$ 10^{-13} cm.$^{-1}$ sec.$^{-1}$ (1). When milks were heated, sedimentation curves showed that the protein particles increased in size, which was dependent on the time and temperature, and it was also affected by the hydrogen ion concentration (4, 5).

The purpose of this investigation was to continue to study the effects of ultra-high temperatures on milk proteins before and after vacuum concentration, with emphasis on changes in storage which lead to gel formation.

EXPERIMENTAL PROCEDURES

Raw whole milks from the University herds were preheated, vacuum-concentrated, and sterilized by ultra-high temperatures in the Mallory small tube heat exchanger (3). They were concentrated either in a stainless steel vacuum pan of about 1,200 lb. finished capacity, in a stainless steel vacuum pan of 120 lb. capacity, or in a sterile glass distillation flask.

Sedimentations were made in a Fisher-Stern Ultracentrifuge and pictures of the sediment were measured (4). It was demonstrated in preliminary trials that the viscosity of concentrated milks had so much influence on the sedimentations that it was impossible to determine the effect of the size of particles. Reconstituted milks yielded sedimentation curves that were comparable to, and in some cases identical with, those for the whole milks before concentration. The

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milks were reconstituted by weighing into 50-ml. volumetric flasks the quantity of concentrated milk necessary to yield the total solids of the original milk at 70° F. The flasks containing the concentrated millks were equilibrated (5) in a room at 70° F. Immediately before ultracentrifugation, deionized water at 70° F. was added to obtain the desired concentration of solids.

It had been observed in many concentrated milks that no cream layer appeared during ultracentrifugation. To investigate the possibility of fat being carried down by a sweeping action, Milk 1 (Table 1) was reconstituted to its normal composition and 80, 60, 40, and 20% of normal solids.

TABLE 1

Effect of heat treatment on sedimentation constants calculated at time of maximum depth of sediment

Milk	Preheating		Homogenizing	Concentrate		Sedimentation constants ($\times 10^{-13}$) Whole millk		Reconstituted	
	° F.	Sec.	° F.	° F.	Sec.	Raw	Pre-heated	From pan or flask	Sterilized
1	285	2.08	285	290	2.08	427		1,160	23,000
2[a]	285	2.08	285	290	2.08	414		820	16,000
3	280	[b]	155	.....		415	835	820	.....
4	280	2.08	280	.....		415	701	760	.....
5	300	[b]	155	.....		415	923	1,340	.....
6	300	2.08	300	.....		415	1,170	1,590	.....
7	300	2.08	155	.....		415	1,031	1,340	.....
8	300	4.16	300	.....		415	1,170	1,340	.....

[a] Skimmilk.
[b] No hold.

The difference between the sedimentation curves of milks reconstituted as they came from the vacuum pan, compared with the same milks reconstituted after they had been sterilized, indicated needed information for sterile milks concentrated under aseptic conditions without subsequent heat treatment. Samples of a milk were sterilized and homogenized under six different conditions (Table 1) and held at 32° F. until they were removed for aseptic concentration at 26.5 in. of vacuum in a laboratory flask. After concentration the samples were stored at 100° F.

A phase microscope was used to study the appearance of particles before and after concentration.

RESULTS

In Figure 1, the sedimentation curves (10,000 G) of raw whole milk (4.3% fat) and the same milk after clarification and standardization to 3.5% fat are compared with the sedimentation curve of centrifugally separated skimmilk from the same milk. Also in Figure 1, the sedimentation curves of Milk 1 (3.5% fat) and Skimmilk 2 (Table 1) are shown after the milks were reconstituted as they came from the pan before sterilization. Sedimentation curves (1,500 G) of the same milks reconstituted immediately after sterilization and after storage seven and 28 days at 70° F. are shown in Figure 2. When Milk 1 was diluted to 80, 60, 40, and 20% of normal after seven days of storage at 70° F. and ultracentrifuged (1,500 G), there was a noticeable cream layer in only the 60%

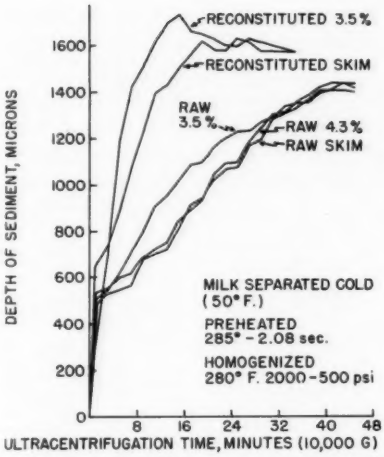

FIG. 1. Sedimentation of raw whole milk, raw skim, raw standardized milk, and same milks after preheating, concentration, and reconstitution before sterilization.

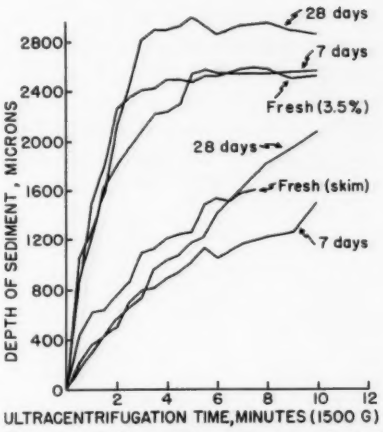

FIG. 2. Sedimentation of reconstituted sterile concentrated Milks 1 and 2 after storage at 70° F.

dilution and it was less than one-fifth of the expected amount for untreated milk at that dilution. The effect of decreased viscosity and density of the more dilute solutions is evident in less time being required to reach maximum depth of sediment (Figure 3).

Sedimentation curves (10,000 G) of whole Milks 3-8 (Table 1) immediately

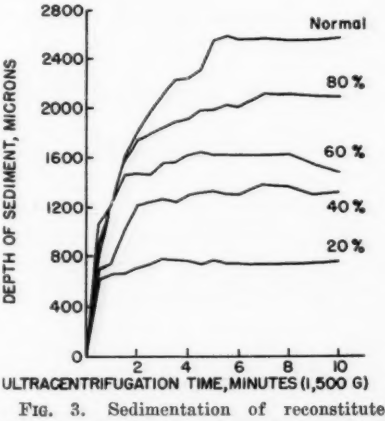

FIG. 3. Sedimentation of reconstituted Milk 1 after storing seven days at 70° F., normal composition and 80, 60, 40, and 20% of normal.

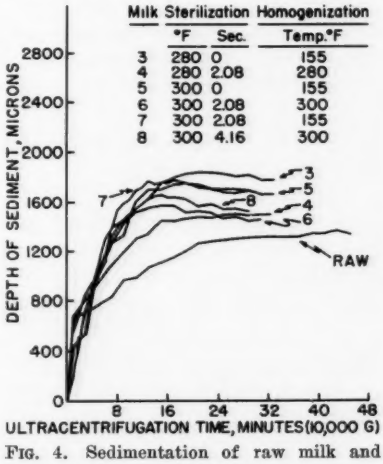
Milk | Sterilization °F | Sterilization Sec. | Homogenization Temp.°F
3 | 280 | 0 | 155
4 | 280 | 2.08 | 280
5 | 300 | 0 | 155
6 | 300 | 2.08 | 300
7 | 300 | 2.08 | 155
8 | 300 | 4.16 | 300
RAW
DEPTH OF SEDIMENT, MICRONS
ULTRACENTRIFUGATION TIME, MINUTES (10,000 G)

FIG. 4. Sedimentation of raw milk and same milk after sterilization.

after sterilization are shown in Figure 4. The time required for gel formation in the aseptically concentrated milks varied from one to 42 days. There was no correlation between controlled factors and time of gel formation. The concentrated milk which gelled in one day (Milk 3) was held an additional 90 days at 100° F. with no evidence of bacterial growth. Milk 4, which gelled after 42 days, was reconstituted and ultracentrifuged after two and ten days of storage at 100° F. These sedimentation curves (10,000 G) are compared with the curve for Milk 4 immediately after sterilization (Figure 5). The decreased

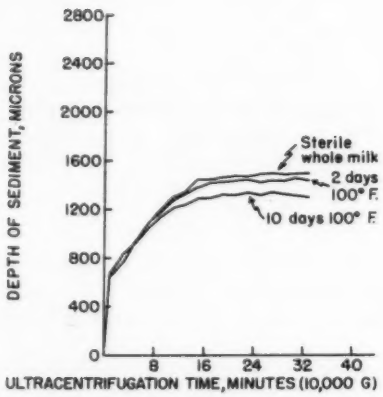

FIG. 5. Sedimentation of Milk 4 immediately after sterilization and reconstituted after two and ten days of storage at 100° F. following aseptic concentration.

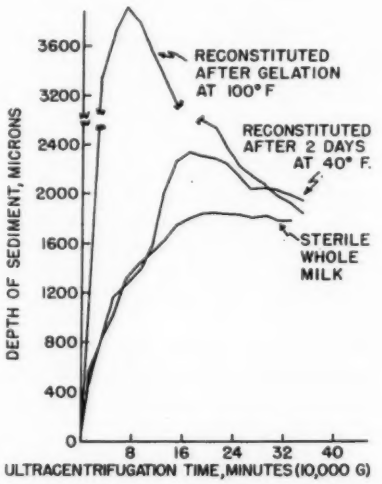

FIG. 6. Sedimentation of Milk 3 immediately after sterilization and reconstituted after two days at 40° and after gelation at 100° F.

volume of sediment was typical of sterile whole milks stored at 100° F. for the same periods of time (5).

In the two samples of Milk 3 which had been concentrated three and 18 days after the milk was sterilized, gel formation was noted in the concentrated milks at 100° F. in 14 days and one day, respectively. Sedimentation curves of freshly sterilized Milk 3, reconstituted after the concentrated milk had been stored 40° F. for two days, and reconstituted after the concentrated milk had gelled at 100° F., are shown in Figure 6. Similar sedimentation curves for Milks 5 and 6 are shown in Figures 7 and 8.

Sedimentation constants (Table 1) calculated at time of maximum depth of sediment (5) did not change greatly after concentration and reconstitution. When the size of particle was calculated from the equation,[2] $d = \sqrt{\frac{18\,\eta s}{\rho - \rho_o}}$, d for

[2] d is diameter; η is viscosity; s is sedimentation constant; ρ and ρ_o are densities of particle and solvent, respectively.

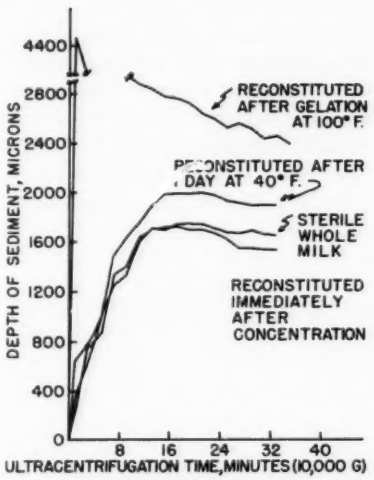

FIG. 7. Sedimentation of Milk 5 immediately after sterilization, reconstituted immediately after concentration, after one day at 40° and after gelation at 100° F.

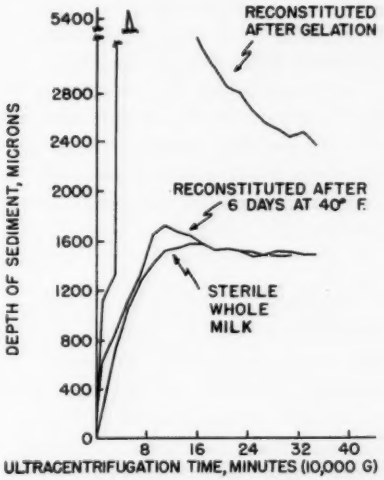

FIG. 8. Sedimentation of Milk 6 immediately after sterilization, reconstituted after six days at 40°, and after gelation at 100° F.

particles having a sedimentation constant of 3,000 was found to be approximately 0.2 μ and for a constant of 30,000 approximately 0.7 μ. Such particles should be visible under a microscope at 450 × magnification. When a reconstituted sterile concentrated milk was examined microscopically, the most common size particles, $d = 2$ μ, appeared to be fat globules surrounded by protein. When this value for d was substituted in the equation, the value for ρ-ρ_0 became 0.1. These particles were counted in a hemocytometer and found to be sufficiently numerous to account for essentially all of the fat, casein, and denatured serum protein in the milk.

Microscopic examination of milks after preheating revealed clustering of particles in those milks which sedimented relatively fast, followed by packing of the sediment layer. In accord with results of sedimentations, very little if any microscopic difference could be found between the preheated milks and the same milks reconstituted as they came from the vacuum pan or flask. The appearance of the milks reconstituted after sterilization was in accord with the relative size changes indicated by sedimentation constants (Table 1). For other milks not reported in detail, calculated sedimentation constants greater than $40{,}000 \times 10^{-13}$ were associated with microscopic clusters of particles rather than further increases in the size of the individual particles. A few of the largest particles appeared to be fragments of leucocytes or bacteria covered with a layer of protein.

During storage of concentrated milks at 70 and 100° F., the first change noted was an increase in the number of smaller particles. This was followed by particles joined together in two's and three's, then in larger groups and finally

a network, or spongelike structure. At this point the gel structure was evident in the milks and the sedimentation constant for the reconstituted milk (Figure 7) was 114,000 $\times$ 10^{-13} for Milk 5. Other milks did not reach maximum depth of sediment as quickly as Milk 5 (Figures 6, 8). In all such sedimentations, the maximum depth of sediment did not represent complete sedimentation of all the particles sedimentable at the given velocity. Visual observation of the cell during sedimentation revealed smaller particles moving toward the sediment as the sedimented layer packed.

DISCUSSION

The type of ultracentrifuge used in these studies was well adapted because of the short time required to reach desired velocity (4) and the calculation of sedimentation constants from zero time. Any errors due to velocity control or photography were reflected in irregularities in the curves, but packing of the sedimented layer also was evident in varying degrees and contributed to the irregularities. The limitations due to packing should be considered in using these constants. Constants calculated for other milks (unpublished data) have varied more than the results reported in Table 1. For raw milks, they have varied from 370 to 440 $\times$ 10^{-13} cm.$^{-1}$ sec. $^{-1}$. In these studies, it has not been possible to demonstrate particles as small as the smallest micellar particles reported by Ford and Ramsdell in which $s = 180 \times 10^{-13}$ (1).

Sedimentation constants for whole milks heated to 320° and milks heated to 180° for 10 or 30 min. before being heated to 300° F., have been about 3,000 $\times$ 10^{-13}. Considerable clustering of protein particles has been evident in microscopic examination of these milks. This type of clustering, which was associated with increased viscosity, was different from the type of clustering involved in gelation.

These differences are in accord with King's (2) description of "Numerous flocks of loosely aggregated particles in some brands of evaporated milks" as contrasted to rennet action which "suggests a partial unfolding of the casein molecules with the formation of tails." The appearance under the microscope in the early stages of the gel structure was that of the opening of definite reactive sites which combined with other particles in a characteristic pattern. However, the tail with the space between particles, as described by King, was not found in these studies except in one case in which bacterial contamination was evident. The characteristic pattern was that of discrete particles joined tangentially in two's and three's and straight chains up to six. This was true for not just a few particles but was uniform over the entire field for both whole milk and skimmilk concentrates. Larger clusters and, ultimately, the spongelike structure appeared to be made up of these smaller straight chains.

Denaturation of serum proteins is involved in the increased size of protein particles by heat, but the great change in sedimentation due to application of ultra-high temperatures to the concentrated milks was found in milks which had been preheated sufficiently to denature all of the heat-denaturable serum proteins. When concentrated whole milks were sterilized by ultra-high tempera-

tures, all of the fat globules, provided they were homogenized efficiently, collected enough protein layer to sediment.

A comparison of Figures 2, 6, 7, and 8 and the confirming microscopic appearance, indicated that only about a third of the fat globules collected enough protein to cause them to sediment when sterilized as whole milk. This portion of the fat sedimented did not change due to vacuum concentration. In Milk 3, which gelled in one day, the portion of the fat which sedimented from the reconstituted milk was approximately the same as the milk before concentration. In Milks 5 and 6, which gelled in seven and ten days of storage at 100° F., all of the fat sedimented when reconstituted after gelation. This indicates that seven to ten days at 100° F. was sufficient time for the fat globules to adsorb enough protein to cause them to sediment.

This type of phenomenon, together with the fact that untreated raw milk contained a few particles as large as the most common size in sterile concentrated milk, suggested a relationship between the concentration of the proteins and change in particle size, whether it be an increase in the size of existing protein particles or the aggregation of protein about fat, or foreign materials such as fragments of leucocytes and bacterial cells.

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FATTY ACID COMPOSITION OF THE MONOGLYCERIDES FROM LIPOLYZED MILK FAT

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SUMMARY

The monoglycerides from eight samples of lipolyzed milk fat were converted to allyl esters and identified by gas-liquid chromatography. Fresh milk fat did not contain monoglycerides detectable by the methods used. The majority of the samples contained butyrate through stearate and oleate. Many contained C_{10}-C_{16} monounsaturates, C_9-C_{17} saturates, and linoleate, and up to eight additional unidentified esters. The fatty acids present in largest quantity, reported as per cent by weight of total allyl esters, were: palmitate—31.2, oleate—17.7, myristate—15.6, caprate—5.7, palmitoleate—5.6, linoleate—4.1, laurate—4.0, and stearate—3.9. The patterns were distinctly different from the reported fatty acid composition of intact milk fat.

Monoglycerides have been found in lipolyzed milk fat, but as yet the component fatty acids have not been identified (7, 8). Identification of the fatty acids as methyl esters by gas-liquid chromatography (GLC) requires prior isolation of the monoglycerides in pure form. Monoglycerides can be concentrated by extracting a heptane solution of the fat with 80% ethanol (1), followed by silica gel column chromatography of the 80% ethanol fraction (4, 14). However, using these methods, the authors found the monoglycerides from lipolyzed milk fat to be about 70% pure and noted a preferential loss of some monoglycerides.

The difficulties above were avoided in the method of McInnes *et al.* (12), in which 100 mg. or less of monoglycerides can be analyzed for fatty acid composition in the presence of tri- and diglycerides. The total monoglycerides were converted to allyl esters which were subsequently identified and measured by GLC. It was, therefore, reasoned that a preliminary concentration of monoglycerides from lipolyzed milk, coupled with the allyl ester method, would identify the monoglycerides therein. This study reports the application of the methods above to the identification of the monoglycerides from lipolyzed milk fat.

EXPERIMENTAL PROCEDURES

Lipolyzed milk samples were prepared by mixing raw and homogenized milks, obtained from the University mixed supply during a 2-mo. period, in equal portions and storing the mixture at 4° C. for 48 hr. The fat was then extracted with chloroform-methanol (90:10) from 500 ml. of each sample by the silica gel method (3). The solvent was removed with a flash evaporator and an ether solution containing the extracted fat was passed through an ion exchange resin, IRA 400, to remove unesterified fatty acids (1). The ether solution was dried with anhydrous sodium sulfate and the ether removed with a flash evaporator. The fat, dissolved in heptane (20% by weight), was par-

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titioned between heptane and 80% ethanol (1). Determination of the monoglyceride contents of the extracted fat, the 80% ethanol fractions, and the heptane fractions indicated that an average of 93.5% of the total monoglycerides appeared in the ethanol fractions (8). It was found that the concentrations of monoglycerides in the 80% ethanol fractions (50-90 m*M* per 100 g. fat) were sufficient for utilization of the allyl ester method. Consequently, the method was subsequently applied only to the 80% ethanol fraction. The resultant esters from each of eight lipolyzed fat samples were analyzed by gas-liquid chromatography, using a Leeds-Northrup recorder and an Aerograph Model A-90-C maintained at 195° C. Control samples of fresh milk were extracted and the lipids were worked up as indicated above. These samples did not yield any peaks when injected into the gas-liquid chromatograph. Separations were obtained on columns of butanediol succinate and diethylene glycol succinate polyesters supplied by the instrument manufacturer.[1] Peaks were identified by comparison with the relative retention times of known allyl esters, either synthesized from pure monoglycerides, obtained as a gift, or obtained from a commercial source.[2] The areas under the peaks were estimated by triangulation. Although accompanying tri- and diglycerides do not interfere in the GLC separation, they probably decrease column life. Therefore, the allyl ester–80% ethanol fraction mixtures were purified by silica gel column chromatography (14), in which the allyl esters were eluted with benzene. The procedure was checked by silica gel chromatography of known mixtures and found to provide almost complete recovery of allyl esters, as well as to remove diglycerides and some triglycerides.

RESULTS AND DISCUSSION

The fatty acid composition data of the monoglycerides are presented in Table 1. The fatty acids are designated by the shorthand method used by Farquhar *et al.* (2), in which butyric acid is 4:0, where 4 represents the number of carbons and 0 the number of double bonds, and oleic acid is 18:1. Oleic, palmitic, and myristic acids were present in the largest amount, comprising together about 64.5% of the total. The fatty acid patterns of the monoglycerides were quite different from those reported for intact milk fat. The concentrations of butyrate, stearate, and oleate were much lower and myristate was higher in the monoglycerides than in intact fat as determined by GLC (13). All of the fatty acids listed have been shown to be present in milk fat (5, 15). However, the concentrations of some of the fatty acids, notably 10:1, 12:1, 14:1, 15:0, 16:1, and 17:0, were higher in the monoglycerides than the concentrations reported for milk fat. Most of the fatty acids known to occur in milk fat were found in the 2-monoglycerides. Exceptions were 20:0 and higher, and some of the 18-carbon polyunsaturates. The apparent absence of these fatty acids might well be due to inadequacies of the GLC method as used herein. The results, except for oleate, myristate, and stearate, agreed

[1] Wilkens Instrument and Research, Inc., P.O. Box 313, Walnut Creek, California.
[2] K and K Laboratories, Inc., 177-10 93rd Avenue, Jamaica 33, New York.

TABLE 1

Gas chromatographic analysis of allyl esters of monoglycerides from lipolyzed milk fat

Fatty acid		Percentages of total allyl esters								
		Samples								
No.	Shorthand [a] designation	1	2	3	4	5	6	7	8	Means
1.	4:0	0.2	....	1.1	1.1	0.5	....	0.9	1.9	1.0
2.	Unknown	0.2	0.9	0.9	....	....	....	0.2	....	0.6
3.	6:0	1.1	0.5	....	7.6	0.4	....	0.1	0.5	1.7
4.	Unknown	0.8	....	....	....	....	....	1.2	0.2	0.7
5.	Unknown	0.8	....	....	....	....	....	....	....	0.8
6.	8:0	0.7	1.1	1.0	3.2	0.4	1.5	0.7	0.9	1.2
7.	9:0	0.7	0.5	....	4.0	....	....	....	0.3	1.4
8.	Unknown	....	....	1.1	....	....	....	0.2	....	0.7
9.	10:0	2.3	2.9	8.0	9.4	17.6	1.5	1.5	2.2	5.7
10.	10:1	2.0	1.1	....	3.2	1.4	....	0.2	0.9	1.5
11.	11:0	....	....	0.2	....	0.7	0.1	....	0.1	0.3
12.	12:0	2.3	6.3	1.9	5.0	2.5	6.2	4.3	3.6	4.0
13.	12:1	2.3	0.9	....	....	0.4	0.5	0.4	1.4	1.0
14.	13:0	0.2	0.7	....	....	....	....	....	0.2	0.4
15.	Unknown	....	....	....	....	5.0	....	0.3	0.2	1.8
16.	14:0	11.8	26.7	6.7	18.7	8.7	20.0	17.5	14.9	15.6
17.	Unknown	....	....	....	....	3.8	....	....	....	3.8
18.	14:1	6.5	3.3	0.4	....	1.6	3.0	4.7	2.3	3.1
19.	Unknown	....	....	....	....	....	....	....	3.4	3.4
20.	15:0	1.3	2.6	5.4	....	0.8	1.7	1.4	1.4	2.1
21.	16:0	29.8	37.9	43.2	26.5	23.4	25.5	34.6	28.6	31.2
22.	16:1	2.9	3.7	2.1	2.5	2.4	16.3	6.0	8.7	5.6
23.	17:0	3.6	....	....	....	0.7	1.0	1.3	1.6	1.6
24.	Unknown	0.4	....	....	....	....	....	0.6	0.5	0.5
25.	18:0	5.0	2.4	6.2	2.9	....	3.0	4.2	3.9	3.9
26.	18:1	17.5	8.5	21.8	15.0	25.4	15.7	19.0	18.4	17.7
27.	18:2	7.6	....	....	....	4.3	4.0	0.7	3.9	4.1
Monoglycerides *(mM/100 g. fat)*		2.30	0.90	1.12	2.55	1.65	2.56	2.25	2.77	

[a] The first figure refers to the number of carbons and the second to the number of double bonds.

reasonably well with those reported by Patton *et al.* (13) for monoglycerides from milk fat digested with pancreatic lipase. The data presented herein do not show if milk lipase hydrolyzes specific fatty acids. Since milk lipases preferentially hydrolyze the primary ester linkages of triglycerides (9), the remaining fatty acids on the 2-monoglycerides can yield little information concerning fatty acid specificity. The free fatty acids and diglycerides would also have to be recovered and the component fatty acids determined to ascertain this type of specificity. Nevertheless, it is obvious that the allyl ester method would be extremely useful in a study of lipase specificity and also of triglyceride structure.

Some of the fatty acids reported in Table 1 may well be artifacts or products of oxidation, e.g., the presence of pelargonic acid in some of the samples supports this. The area under some of the peaks may have been altered by interesterification of the allyl esters with the liquid phase in the column (11). However, when the allyl esters were separated on two different columns, the relative percentages were remarkably constant. McInnes *et al.* (12) found that 8:0 and higher allyl esters could be determined quantitatively by GLC. Since the monoglycerides can be quantitatively extracted from milk (6) and extracted

with 80% ethanol from a heptane solution of the fat, it appears that the data for 8:0 and higher acids in Table 1 are reliable. The allyl esters of 4:0 and 6:0 were present, but the amounts of these esters shown in Table 1 may be uncertain because of errors in measurement of the area under peaks of such low retention times. Nevertheless, the low concentration of butyric acid in the 2-monoglycerides, compared to that reported for intact milk fat, agrees with the findings of Kumar and Lalka (10), who digested milk fat with pancreatic lipase and found that a large fraction of the butyric acid in milk triglycerides is located in the primary positions.

ACKNOWLEDGMENTS

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SPECIFICITY OF MILK LIPASE TOWARD THE PRIMARY ESTER GROUPS OF SOME SYNTHETIC TRIGLYCERIDES[1]

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SUMMARY

Two symmetrical triglycerides, 2-oleoyl dipalmitin and 2-palmitoyl diolein, were synthesized and then subjected to hydrolysis at pH 6.6 by milk lipase. The results indicate that milk lipases preferentially hydrolyze the primary hydroxyl esters of synthetic long-chain triglycerides.

As yet, few data have appeared concerning the positional specificity of milk lipase for triglycerides. Previous reports indicated, however, that milk lipases exhibit certain specificity for the primary hydroxyl esters of tributyrin (4) and for the primary hydroxyl ester positions of milk triglycerides (6).

Jensen *et al.* (6) indicated that final proof of position specificity would involve the lipolysis of symmetrical triglycerides with a different fatty acid in the 2-position. Mattson and Beck (9, 10), using several symmetrical triglycerides and 1-oleoyl dipalmitin as substrates, have proven that pancreatic lipase specifically hydrolyzes the primary hydroxyl groups of triglycerides. They recovered the free fatty acids, mono-, and diglycerides and identified the constituent fatty acids by means of iodine numbers.

Since milk is much lower in lipase activity than pancreatic lipase preparations, the quantities of lipolytic products are too small for iodine number determination. Gas-liquid chromatography, on the other hand, makes it possible to handle minute quantities of material. Because of the sensitivity of the gas-liquid chromatograph, however, it is necessary for the triglyceride to be of high purity. Mixed symmetrical triglycerides are not available commercially; consequently, laboratory syntheses of these compounds were undertaken.

This paper describes a study in which 2-oleoyl dipalmitin and 2-palmitoyl diolein were synthesized and then hydrolyzed by milk lipases. The products of lipolysis were recovered by silica-gel column chromatography and identified by gas-liquid chromatography.

EXPERIMENTAL PROCEDURE

Enzyme Source. Fresh, raw warm milk collected from the University herd was centrifuged to remove fat and the skimmilk lyophilized. Several batches of raw skimmilk powder were prepared until about 500 g. was obtained. This material was pooled, carefully mixed, and stored at −20° C. This skimmilk powder was used as the enzyme source throughout these experiments.

Triglyceride synthesis. The two triglycerides, 2-oleoyl dipalmitin and 2-palmitoyl diolein, were synthesized from the corresponding 2-monoglyceride

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by acylation with the appropriate fatty acid chloride in the presence of pyridine as a catalyst. Oleoyl chloride was prepared with oxalyl chloride (1) and highly purified oleic acid obtained from the Hormel Foundation. The 2-monoglycerides were prepared from 1,3-benzylidine glycerol according to the procedure of Martin (8). The monoglycerides were over 95% 2-monoglyceride as determined by the method of Jensen and Morgan (7). The 1,3-benzylidine glycerol was synthesized essentially by the method of Verkade and Van Roon (14), as modified by Porck and Craig (11).

The triglycerides prepared above were contaminated with small amounts of free fatty acids, diglycerides, and monoglycerides. The free fatty acids were removed by passing a wet ether solution of the lipid through an IRA 400 anion exchange resin (9). Mono- and diglycerides were removed by silica-gel column chromatography (12). As determined by silica-gel chromatography (12), the purity of the resulting triglycerides was over 90%. Digestion of the triglycerides with pancreatic lipase showed both triglycerides to be of the proper fatty acid configuration (9).

Digestion conditions. The triglycerides were incubated with pancreatic lipase (steapsin) following the procedure of Mattson and Beck (9). In the experiments with milk lipase the following conditions were used: Three grams of raw skimmilk and 1 g. of triglyceride were made up to a total volume of 25 ml. When necessary, the pH was adjusted to 6.6. The substrate was emulsified into the raw skimmilk solution with a Waring Blendor. A control was prepared in the same manner, except that the skimmilk solution was heated to 70° C. for 5 min. before addition of the substrate. These samples were then incubated at 37° C. with shaking for 2 hr.

Analysis of hydrolytic products. After incubation, the reaction mixture was adjusted to a pH of 2 with 20% H_2SO_4, then mixed with silicic acid and extracted chromatographically with ethyl ether essentially as described by Harper *et al.* (5). The ether solution containing neutral fat and fatty acids was passed through a column of IRA 400 anion exchange resin to remove the free fatty acids. The acids were subsequently eluted with 10% acetic acid in ether, the solvent was evaporated with a flash evaporator, and the residual fatty acids were methylated with methanol and dimethoxy propane (13). The eluate from the anion exchange column contained the neutral lipids which, after removal of the solvent, were applied to silicic acid columns and separated into tri-, di-, and monoglycerides (12). The resulting di- and monoglyceride fractions, after removal of solvent, were transesterified with sodium methoxide (2).

The fatty acid composition of each fraction was determined by gas-liquid chromatography of the methyl esters, using an Aerograph Model A-90-C operated at 195° C. with a 5-ft. column of diethylene glycol succinate on Chromosorb. The relative concentration was determined by measurement of the area under each peak in the gas-liquid chromatogram (3).

RESULTS AND DISCUSSION

The triglyceride 2-palmitoyl diolein was subjected to milk lipase action for 2 hr. at pH 6.6. The products of lipolysis were isolated and the fatty acid

composition determined. This experiment was repeated four times, and the average values obtained are given in Table 1. The results show that 90% of the free fatty acids was oleic, indicating that the fatty acid in the primary position (oleic) was preferentially hydrolyzed. If the enzyme exhibited no specificity for the primary position, then one would expect a yield of 66% oleic acid and 33% palmitic acid. The diglycerides formed contained almost equal amounts of palmitic and oleic acids, which would have been expected if the enzyme were specific for the primary alcohol ester groups. The monoglyceride

TABLE 1
Fatty acid composition of the hydrolytic products of 2-palmitoyl diolein digested with milk lipase

Product	Mole per cent composition [a]	
	Palmitate	Oleate
Free fatty acid	10	90
Diglyceride	51	49
Monoglyceride	88	12

[a] Average of four trials.

fraction was shown to be predominantly monopalmitin; and this further substantiates the specificity of the enzyme for the primary positions with the synthetic substrates used.

These results could also be interpreted as indicating specificity of the enzyme for oleic acid rather than for the primary position of the triglyceride. In order to show whether milk lipase exhibits specificity for oleic acid or for the primary hydroxyl ester, 2-oleoyl dipalmitin was subjected to milk lipase action at pH 6.6 for 2 hr. The fatty acid composition of the products is shown in Table 2. This experiment was performed only twice, because of lack of

TABLE 2
Fatty acid composition of the hydrolytic products of 2-oleoyl dipalmitin digested with milk lipase

Product	Mole per cent composition [a]	
	Palmitate	Oleate
Free fatty acid	91	9
Diglyceride	48	52
Monoglyceride	16	84

[a] Average of two trials.

substrate. In this case, the predominant free fatty acid was palmitic, the diglycerides contained almost equal amounts of palmitic and oleic, and the monoglycerides were mostly oleic. These results confirm the conclusion that milk lipase shows specificity for the primary hydroxyl esters of synthetic triglycerides. In this respect milk lipase is similar to pancreatic lipase.

In all of the experiments some of the fatty acid in the 2 position appeared to be hydrolyzed and the monoglyceride fraction contained some fatty acid which should appear only in the 1 or 3 position, a phenomenon noted in the previous investigation (6). Because of the long incubation and slightly acid

medium, acyl group migration could account for the results; however, milk lipase may hydrolyze the secondary alcohol ester to some extent. A more concentrated enzyme preparation, with correspondingly shorter incubation times, is necessary in order to distinguish between these two possibilities.

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INFLUENCE OF ALFALFA AND OAT HAYS ON SUSCEPTIBILITY OF MILK TO OXIDIZED FLAVOR

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SUMMARY

Alfalfa and oat hay were compared as to influence on the susceptibility of milk to oxidized flavor. Eight cows were used in a double-reversal design with three 6-wk. periods. Milk samples collected twice weekly (treated with 0, 0.1, and 1.0 p.p.m. of added copper and stored under refrigeration two and five days) were examined organoleptically and by the thiobarbituric acid (TBA) test. Copper and citrate were determined weekly. Fat samples churned weekly from the milks were examined for oxidative stability (induction period), tocopherol, carotene, iodine value, and individual polyunsaturated fatty acids.

On the basis of flavor scores, TBA tests, and oxidative stability, milk from oat hay was much more resistant to oxidation than milk from alfalfa hay. Mean respective values for alfalfa and oat rations, together with standard error of differences, were: copper (γ/g), 0.090, 0.057, 0.006; citrate (mg/g), 2.7, 2.6, 0.05; total tocopherol (γ/g), 13.7, 32.0, 2.5; total carotenoids (γ/g), 5.0, 2.1, 0.7; and iodine value, 27.1, 29.8, 1.3. The polyunsaturated fatty acids in fat were higher from alfalfa hay than from oat hay. Differences between the means for most of these variables were statistically highly significant, but additional work is needed to determine the relative importance of the various constituents in determining susceptibility to oxidation.

Rations consisting mainly of alfalfa hay and a concentrate mixture are in common use on California dry-lot dairies. Milk produced on such rations is frequently highly susceptible to oxidized flavor.

In general, milk produced on rations that include green succulent feeds and silage is more resistant to oxidized flavor than milk from dry feeds, but there are many exceptions to this broad statement. In some California dairies addition of green-chopped alfalfa to an alfalfa hay–concentrate ration has not corrected the high susceptibility to oxidized flavor. Furthermore, dry feeds vary a great deal in their effects. In a comparison conducted in 1958 (results not published) milk produced on an alfalfa hay ration was highly susceptible to oxidized flavor, but that produced from oat hay was resistant and comparable with milk from cows on a succulent grass pasture.

Literature on the relation of feed to the oxidative stability of milk is reviewed elsewhere (9, 19, 23). Conclusions from selected publications dealing with the constituents included in the present study are summarized below.

The natural copper of milk is an important catalyst of oxidized flavor when cows receive an alfalfa hay–concentrate ration (11). Reports on whether citrate added to milk influences oxidized flavor are inconsistent (17, 18). Riel and Sommer (19) summarized their review of carotene in relation to oxidized flavor as follows: "Feeding carotene to the cow has been reported to decrease the onset of the oxidized flavor. On the other hand, several other

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workers have concluded that carotene content of milk bears no correlation to the susceptibility to oxidized flavor development." Krukovsky *et al.* (12) and others (6, 19) have observed positive relations among the tocopherol contents of feed, and of milk fat, and the resistance of milk to oxidized flavor.

The fatty acid and fat-soluble antioxidant composition of milk fat is influenced by the cow's feed, and in turn influences the oxidative stability of milk fat (21, 22). Constituents in the skimmilk phase, however, may be the most important in determining whether milk develops oxidized flavor (12, 22). The material concentrated at the surface of the fat globules is undoubtedly involved, too, since oxidized flavor in milk is generally attributed to oxidation of unsaturated fatty acids in the membrane phospholipids. Furthermore, substances considered to be catalysts of oxidized flavor, such as copper-protein (11) and xanthine oxidase (2), are concentrated at the interface.

The investigation reported herein was undertaken to compare susceptibility to oxidized flavor of milk produced on rations in which either alfalfa hay or oat hay was the only roughage. Particular attention was directed to changes in composition of the milk with respect to selected constituents that might influence susceptibility to oxidized flavor. Significant differences related to the ration were found in the susceptibility of the milk to oxidized flavor, the natural copper, total carotenoid, and tocopherol contents of the milk, and the oxidative stability and unsaturated fatty acid composition of the milk fat.

METHODS

The animals selected were six Jersey and two Holstein cows, from 3 to 8 yr. old, that had freshened within 4 to 10 wk. of the experiment. They were paired on the basis of breed and freshening date, and those in each pair were randomly assigned to one of two groups. The groups were given test rations for three 6-wk. periods in a double-reversal design (3, 13, 14).

For 2 mo. before the trial, the cows received alfalfa hay, free choice, and concentrates according to production. During the trial the cows were stanchioned twice daily, following each milking, and group-fed hay in the amounts consumed in a 2-hr. period. Concentrates were fed at milking at the rate of 1 lb. per 3.5 lb. of 4% FCM produced, with the amounts adjusted weekly according to the previous week's production.

Both hays were field-cured. The alfalfa hay was of good quality; the oat hay of only fair quality.

The hays and concentrate mix were analyzed for proximate composition (1) and copper (10). The methods used to determine total carotenes and total tocopherol were essentially those of Bro-Rasmussen and Hjarde (4, 5). Total carotenes were extracted and then eluted from a column of dibasic magnesium phosphate with 0.5% diethyl ether in petroleum ether, and were measured at 437 mμ in diethyl ether (8). Alpha-tocopherol and other tocopherols, respectively, were eluted with 2 and 7% diethyl ether in petroleum ether, and were measured colorimetrically. Respective analyses for the alfalfa and oat hays were: crude protein, 17.8 and 6.7%; crude fiber, 26.7 and 26.0%; ether

extract, 2.3 and 3.4%; dry matter, 93.0 and 92.7%; copper, 10.1 and 3.7 γ/g; total carotenes, 10.5 and 4.0 γ/g; and total tocopherol, 38 and 104 γ/g. The concentrate mix was composed of rolled barley, 43%; rolled milo, 16%; wheat mixed feed, 12%; molasses beet pulp, 8%; cottonseed meal, 20%; and salt, 1%; and contained digestible protein, 13%; TDN, 75%; dry matter, 89.9%; copper, 7.1 γ/g; total carotenes, trace; and total tocopherol, 53 γ/g. The tocopherol in the hays was practically all alpha, but in the concentrate only about 40% was alpha.

Milk samples were collected twice weekly at an afternoon milking, using stainless-steel equipment and polyethylene bottles. For flavor examinations, portions of each sample, treated with added copper at 0, 0.1, and 1.0 p.p.m., were stored under refrigeration at 3-4° C., and examined organoleptically by three trained scorers after two and five days (11), and by the thiobarbituric acid (TBA) test (7) after two days. Copper (11), citrate (15, 16), fat (Babcock), and total solids (Mojonnier) contents of the milk were determined weekly. Fat samples churned weekly from the milks were examined for oxidative stability (21), total tocopherol (8), and iodine value (20). Total carotenoids were measured by determining the absorbance at 437 mμ in diethyl ether (against milk fat blank rendered carotenoid-free by Nuchar) and employing an extinction coefficient of 2,040 in the calculations (8). Individual polyunsaturated fatty acids (20) were determined on samples of fat churned from milk produced in the sixth week of each period. Data were analyzed for significance of differences related to rations by the procedure of Brandt (3) and Lucas (13, 14).

RESULTS AND DISCUSSION

Data for feed consumption and milk production during the feeding trial are summarized in Table 1. The lower hay consumption, and hence milk production, on oat hay was probably attributable partly to the lower palatability of the oat hay. The oat ration provided intakes of only about two-fifths as much copper and one-third as much total carotenes as the alfalfa ration, but almost twice as much total tocopherol. In spite of the difference in milk production, there was no difference related to the ration in the fat and solids-not-fat contents of the milk.

TABLE 1

Average daily feed consumption and milk production per cow during the double-reversal trial

	Ration	
	Alfalfa	Oat
Feed consumption		
Hay *(lb.)*	23.0	18.0
Concentrate *(lb.)*	6.6	6.2
Copper *(mg.)*	127	50
Total carotenes *(mg.)*	109	32
Total tocopherol *(mg.)*	482	928
Milk: Production *(lb.)*	28.4	24.9
Fat *(%)*	4.9	5.0
Solids-not-fat *(%)*	9.2	9.1

The results of the analytical determinations made throughout the feeding trial (Figure 1) indicated that about 3 wk. were required as an adjustment period. Therefore, results for the last 3 wk. of each test period were used for the statistical analyses (Table 2).

For milk samples with added copper, differences in susceptibility to oxidized flavor related to the rations were highly significant ($P < 0.001$), when measured by flavor scores and TBA increases (Table 2). A difficulty in treating the flavor data is that the milks from individual cows varied a great deal in susceptibility to oxidized flavor. A single level of added copper for the group of cows did not provide a sensitive test for all the cows in the group. As illustrated by the data in Figure 2, flavor scores for samples without added copper showed a marked difference between feeds for Cow 153, but little for 132, whereas the opposite was true for samples with 1.0 p.p.m. added copper. If data for the most sensitive test (level of added copper) for each cow had

TABLE 2

Averages of the results on milk from the two rations for the last 3 wk. of the test periods

	Ration		Treatment difference [a]	Standard error of difference
	Alfalfa	Oat		
Flavor score [b]				
at 2 days: no copper added	0.3	0.1	0.2	0.7
0.1 p.p.m. copper added	2.4	0.3	2.3	0.2
1.0 p.p.m. copper added	2.7	0.6	2.4	0.1
at 5 days: no copper added	1.1	0.1	1.2	0.4
0.1 p.p.m. copper added	2.5	0.6	2.1	0.2
1.0 p.p.m. copper added	3.2	1.5	1.9	0.2
TBA increase (absorbance)				
No copper added	0.006	0.001	0.003	0.003
0.1 p.p.m. copper added	0.045	0.005	0.048	0.007
1.0 p.p.m. copper added	0.073	0.015	0.068	0.009
Copper *(p.p.m.)*	0.080	0.057	0.019	0.006
Citrate *(mg/g)*	2.7	2.6	0.1	0.05
Fat stability *(hr.)*	81.7	151.3	−68.7	5.0
Total tocopherol *(γ/g fat)*	13.7	32.0	−18.0	2.5
Total carotenoids *(γ/g fat)*	5.0	2.1	3.4	0.7
Iodine value [c]	27.1	29.8	−3.5	1.3
Unsaturated fatty acids [c] *(% of total fat)*				
Nonconjugated				
monoenoic	21.09	26.61	−5.92	1.34
dienoic	1.73	1.57	0.12	0.05
trienoic	0.68	0.36	0.32	0.03
tetraenoic	0.28	0.19	0.08	0.01
pentaenoic	0.25	0.18	0.06	0.02
Conjugated				
dienoic	0.47	0.33	0.13	0.02
trienoic	0.02	0.01	0.01	0.002

[a] $\frac{G_1 - G_2}{4r}$ (13).

[b] Intensity of oxidized flavor: 0, none; 1, questionable; 2, slight; 3, strong; 4, very strong.

[c] Data for last week of each period.

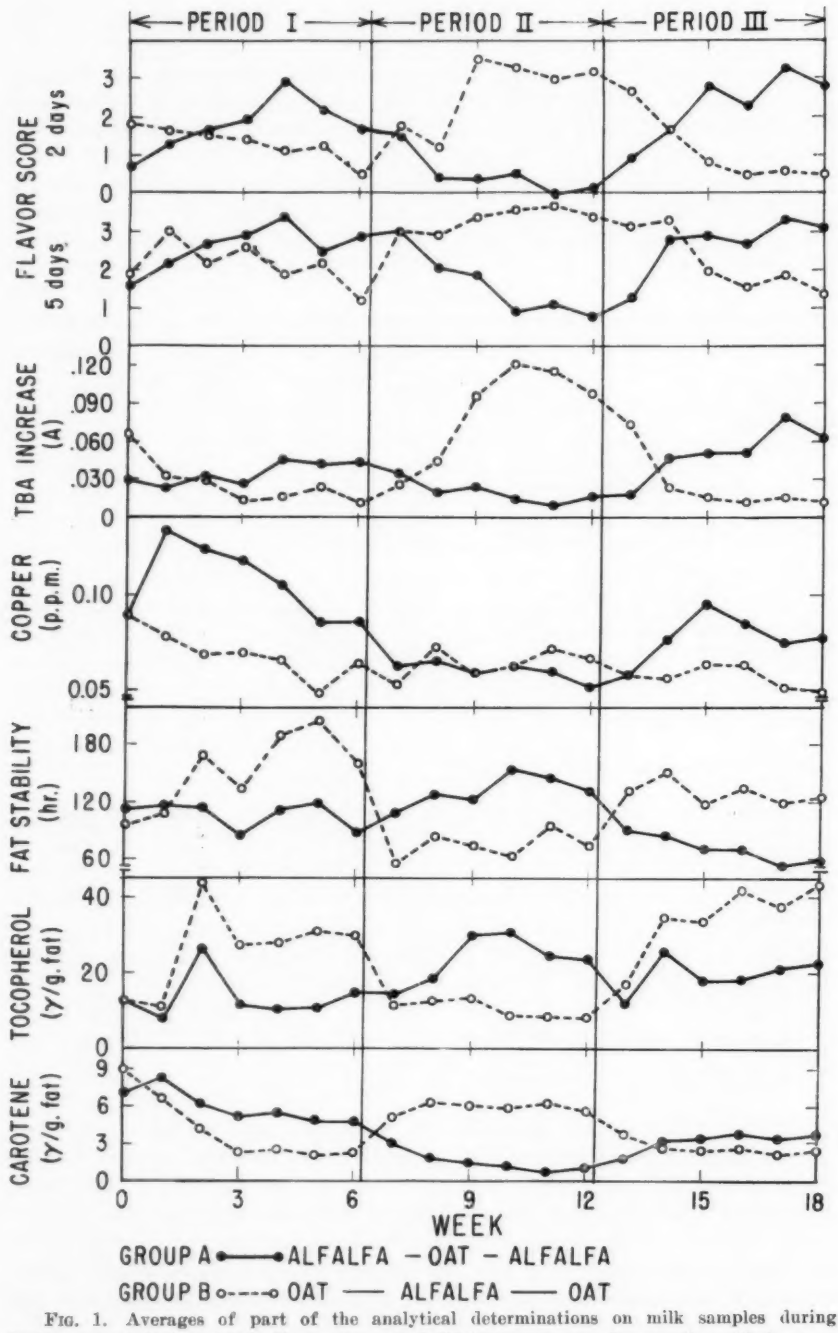

FIG. 1. Averages of part of the analytical determinations on milk samples during double-reversal feeding trial. Flavor scores and TBA increases are for samples with 1 p.p.m. added copper. (Intensity of oxidized flavor: 0, none; 1, questionable; 2, slight; 3, strong; 4, very strong.)

been averaged, differences in flavor scores related to feeds would have been much greater.

For milk samples without added copper, the average differences in oxidized flavor related to the rations were small because of the relatively low incidence of spontaneous oxidized flavor, even on the alfalfa hay ration. Oxidized flavor in milk developed spontaneously in two days from only one cow, and in five days from four cows.

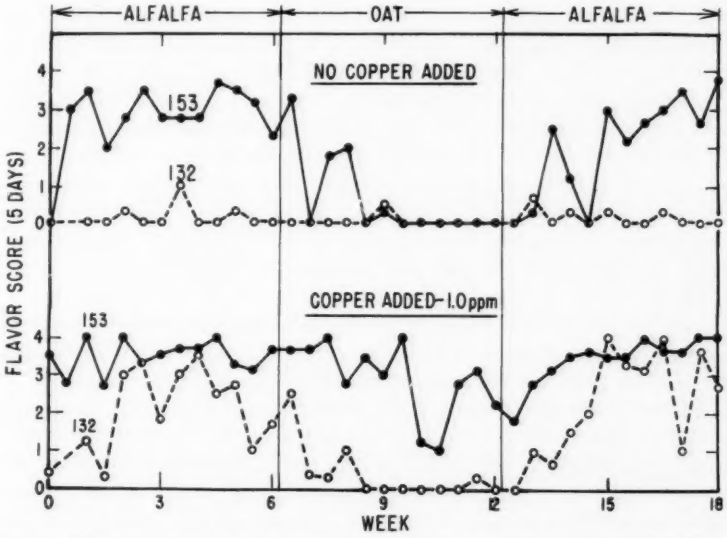

FIG. 2. Flavor scores of milk from two cows on the same ration. These results illustrate variations among individual cows and the influence of the amount of added copper in detecting differences related to feed. (Intensity of oxidized flavor: 0, none; 1, questionable; 2, slight; 3, strong; 4, very strong.)

The difference between the two rations in the natural copper content of the milk was statistically significant ($P = 0.05$). Individual cows varied appreciably in the relation between ration and natural copper content of milk. This varied response is similar to that noted previously (11), when large single doses of copper sulfate were administered by drenching. Interpretation of the data was also complicated by variations in the rate of decrease in the natural copper content of milk during lactation. The higher average copper content of the milk produced on the alfalfa ration, however, was in the direction that would be expected in view of the greater copper intake.

Differences in citrate content of milk were not significant ($P > 0.1$). Since citrate acts synergistically with some antioxidants, possibly by acting as a metal-complexing agent, citrate determinations were included for consideration of whether possible effects of citrate might depend on the copper or tocopherol content of the milk. No such relations were evident.

There was a highly significant difference ($P < 0.001$) in the oxidative stability of milk fat produced on the two rations. The total tocopherol content of the fat was higher from oat hay ($P < 0.001$), and total carotenoids were higher from alfalfa ($P < 0.01$).

The iodine value and oleic acid content were significantly higher in fat produced on oat hay ($P = 0.05$ and 0.01, respectively), but the polyunsaturated fatty acids were significantly higher in the alfalfa milk fat (P from 0.05 to < 0.001). Since polyunsaturated acids are much more labile than oleic acid, it is possible that the minor differences in the amounts of the polyunsaturated acids could be more important in determining the stability of the milk fat and milk than the relatively large difference in the oleic acid contents.

Composition differences related to the ration were highly significant in relation to total tocopherol, total carotenoids, and most of the polyunsaturated fatty acids. It can not be concluded, however, that these differences are important in determining oxidative stability. Nevertheless, in most cases the differences were in the direction that would be expected to give greater stability on the oat hay ration. This is particularly true for the copper and total tocopherol contents of milk and the polyunsaturated fatty acid composition of the fat.

Because of practical limitations, this study did not include measurement of some constituents of feeds that might influence oxidized flavor development, such as ascorbic acid and trace elements other than copper.

In designing the comparison, consideration was given to including tests that would indicate whether changes in susceptibility to oxidized flavor were influenced by changes in the skimmilk, the milk fat, and/or the material concentrated at the fat globule surface. Determination of the oxidative stability of milk fat served this purpose adequately for the milk fat, but no corresponding tests that could be performed routinely were available for the skimmilk and adsorbed membrane fractions. The fraction(s) in which the most important changes occur probably varies with the rations being compared. In this trial, changes in the fat fraction appeared to be important, but the opinion of the authors is that differences in the natural copper content also played a part in the difference in susceptibility to oxidized flavor.

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HEAT RESISTANCE OF THREE STRAINS OF PSYCHROPHILIC ORGANISMS ADDED TO SKIMMILK FOR COTTAGE CHEESE MANUFACTURE

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SUMMARY

The thermal resistance characteristics of *Pseudomonas viscosa, Pseudomonas fluorescens,* and *Pseudomonas fragi* were determined in sterile skimmilk at 110, 115, and 120° F. The thermal resistance of each organism was completely characterized by *D* and *z* values calculated from survivor curve data. *P. fragi* was more heat-resistant than the two other organisms. The results obtained provided evidence that these microorganisms would not survive the normal pasteurization of milk or skimmilk. Furthermore, the survival of these microogranisms during the different stages of making Cottage Cheese was determined. The following conclusions can be drawn from this investigation: (a) These microorganisms do not survive a cooking process of 120° F. for 30 min.; (b) generation of these organisms during incubation is not a problem, because the acidity developed by the time of cutting the curd, produces a 90% reduction in count; (c) none of the species of *Pseudomonas* added to the skimmilk before it was made into cheese could be isolated from the creamed cheese during a storage period of 4 wk. at 38° F.

The importance of psychrophilic bacteria in affecting the keeping quality of dairy products was recognized by Bischoff (1) and Conn (6) as early as 1903. Despite this long acquaintance with information about their properties, dairy bacteriologstis find that the microorganisms responsible for producing the greatest percentage of spoilage in Cottage Cheese under present marketing conditions are psychrophiles—notably bacteria of the genus *Pseudomonas,* yeasts, and molds.

Recommendations for control of these microorganisms have been, in general, of two forms: first, prevention of the original entrance of these microorganisms into the product by knowledge of the sources of contamination; second, prevention of their growth by knowledge of the physiological properties. As applied to Cottage Cheese manufacture, the first method of control is achieved through pasteurization (13, 15, 18, 20), strict plant sanitation practices (8, 15, 17), and treated water supplies (4, 11, 15). The second method of control has been secured through use of low storage temperature (2, 3, 7), control of pH (2, 3), use of chemicals such as sorbic acid (2, 10), acidified dressing for the curd (14), and controlled air supply in the package (19).

While the preponderance of evidence suggests that psychrophiles do not withstand conventional pasteurization procedures (13, 16, 18, 20), it is not equally clear that they do not withstand normal cooking temperatures employed in the manufacture of Cottage Cheese. Therefore, it was considered important to provide the Cottage Cheese manufacturer, who is having a problem

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with psychrophilic spoilage, with specific evidence that the freshly cooked curd is or is not a likely source of these organisms.

The objectives of this investigation were to study the thermal resistance characteristics of three species of *Pseudomonas* known to cause spoilage in Cottage Cheese. These were *Pseudomonas viscosa, Pseudomonas fragi,* and *Pseudomonas fluorescens.* Thermal resistance of these organisms was determined in sterile skimmilk at 110, 115, and 120° F., and also during the process of Cottage Cheese manufacture, by inoculating the fresh pasteurized skimmilk with these organisms at time of setting.

Answers to the following questions were sought:

(a) Do these organisms increase in number during the incubation periods for short-time and long-time setting procedures?

(b) Do these organisms survive the process of cooking Cottage Cheese at 120° F. for 30 min.?

(c) If these organisms do not survive cooking procedures, at what temperature during cooking are they destroyed?

(d) Is the acidity of the whey a factor in determining the survival of these organisms?

(e) Is it possible to recover from the Cottage Cheese, after 2 or 3 wk. of storage at 38° F., any of the *Pseudomonas* organisms used to inoculate the fresh milk at time of setting?

EXPERIMENTAL PROCEDURE

Plating procedure for enumerating Pseudomonas in Cottage Cheese. The three species of *Pseudomonas,* i.e., *P. viscosa, P. fragi,* and *P. fluorescens,* which were used in this study were obtained from Elliker (9). Preliminary testing procedures verified that these organisms could be recovered on plate count (P.C.) agar medium when a mixture of *Pseudomonas* and starter culture were plated and incubated at 42° F. for 14 days. The lactic starter organisms did not develop colonies, whereas the *Pseudomonas* organisms did develop charteristic colonies. This procedure assured that the starter organisms would not be mistaken for *Pseudomonas* when recovery tests were made at the various steps in the Cottage Cheese process.

The thermal resistance of three species of Pseudomonas. Heat sensitivity trials were conducted for the three test organisms. These studies were made at three temperatures, 110, 115, and 120° F. The single tube technique was used.

Samples of the test organisms were prepared by adding 1 ml. of a 24-hr.-old culture to 200 ml. of reconstituted sterilized skimmilk and mixing in a sterilized Waring Blendor for 1 min. An attempt was made to have more than 1,000,000 organisms per milliliter as the initial count in the sample before heating. Five milliliters of the mixed skimmilk was added to sterilized 16- by 150-mm. tubes and capped by Morton closures. Although care was exercised not to wet the sides when adding the sample, flaming of the tubes was done as an extra precaution. All tubes were placed in a wire basket, immersed in a preheating bath, and timing started. Since only 20 to 25 sec. were required

as an initial exposure before the samples reached the required predetermined temperature, the come-up time did not affect the slope of the survivor curve. The basket was then immediately transferred to the constant temperature water bath which was maintained at the selected temperature for the desired exposure period. At definite intervals of time, i.e., 1 min., 2 min., etc., throughout the entire exposure period, samples were quickly removed from the water bath and immediately placed in an ice bath. The samples were plated immediately, using P.C. medium, incubated at 72° F. for three days, and counted. Survivor curves were constructed from the data obtained. All test samples were plated in duplicate. Therefore, all counts reported in tables represent the average of duplicate plates.

The destruction of Pseudomonas organisms during the manufacturing process of Cottage Cheese. The thermal resistance characteristics of the test organisms were also determined in an environment that would be used for Cottage Cheese manufacture. The experimental lots of Cottage Cheese were made from milk of the University dairy herd. This was mixed from various dairy breeds. It was separated and the skimmilk was pasteurized at 143° F. for 30 min., cooled to 40° F., and stored overnight at 38° F. The cream was standardized in 30-lb. lots to 13% fat and pasteurized at 143° F. for 30 min. The following morning the dressing was repasteurized at 175° F. for 20 min., cooled to 90° F., and homogenized at 2,000-lb. pressure on one valve. The dressing was then cooled over a surface cooler, which had been previously sterilized with a chemical sterilizer, and stored in a sterilized stainless steel can at 38 to 40° F. until used. This was generally 3 to 4 hr. after processing.

Pasteurized skimmilk in approximately 20-gal. lots was used in making three lots of Cottage Cheese by the short-time method and one lot by the long-time setting method. To each lot of skimmilk, 100 ml. of the test organism was added for each batch of cheese which produced a range of 500,000 to 9,000,000/ml as an original inoculation of the *Pseudomonas* organisms. The Cottage Cheese was made according to accepted manufacturing procedures. The curd was cut when the pH was 4.7 and the titratable acidity was approximately 0.5-0.52%. The curd was cooked to 130° F. in 2 to 2.5 hr. and the whey was then drained. Wash water was added in three portions at 20-min. intervals after draining the whey.

Ten parts per million chlorine was added to each lot of water and held for 20 to 60 min. before use. The pH of the final rinse was adjusted to 4.8 to 5.0 by adding HCl (C.P.) before adding the water to the cheese. The pH of the freshly creamed cheese was approximately 5.1.

Cheese samples were packaged in sterilized containers and were stored at 38, 50, and 60° F., for various periods of time. These storage samples were again examined for the development of psychrophilic organisms after 1-, 2-, and 4-wk. intervals.

RESULTS AND DISCUSSION

Results of the investigation will be discussed in three parts.

Thermal resistance of the three test organisms. Survivor curves were deter-

mined for each of the organisms from the results of controlled heating studies at the following temperatures: 110, 115, and 120° F. A best straight line was constructed from the experimental points for each survivor curve, using the method of least squares. All values on a curve, except those at the end of a run which were clearly nonlogarithmic, were used in the calculations. A survivor curve for *P. viscosa* is illustrated in Figure 1 and is representative of those obtained for the other organisms. To characterize the slope of the sur-

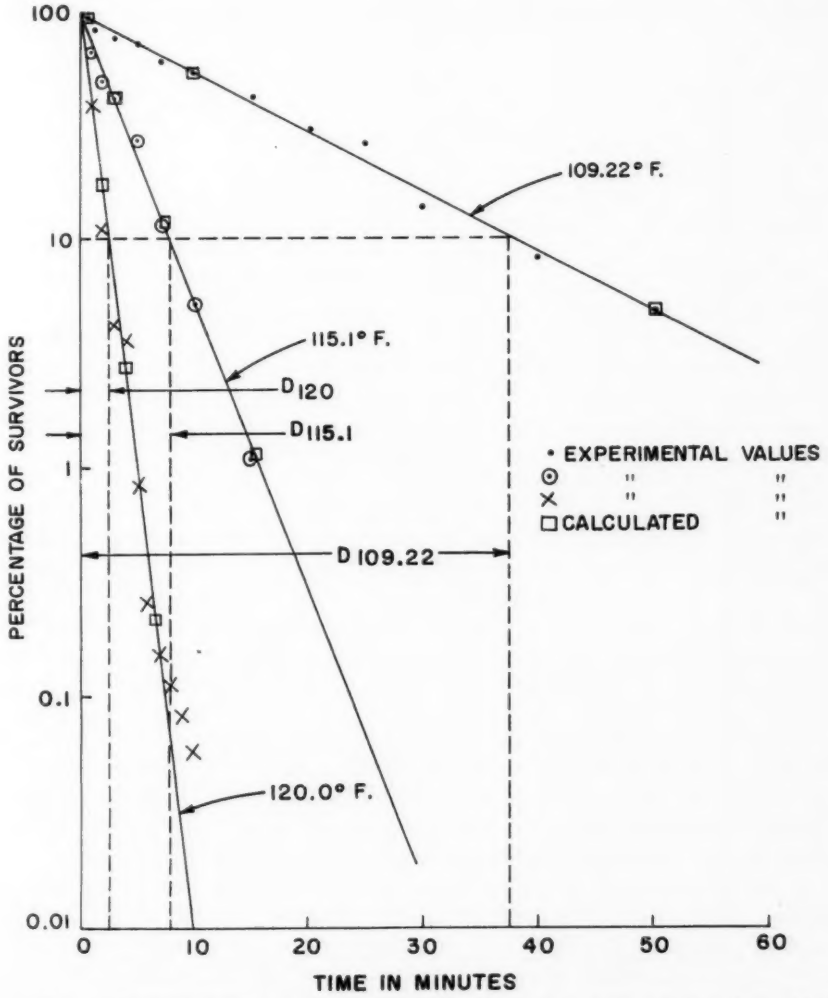

FIG. 1. Survivor curves for *Pseudomonas viscosa* at three temperatures and graphical representation of *D* values at those temperatures.

vivor curve, the decimal reduction time or D value was used (5). A thermal resistance curve for each organism was also constructed by plotting D values on a logarithmic scale versus temperature (° F.) on a linear scale. The slope of the thermal resistance curve is characterized by a z value, which is the degrees Fahrenheit required to secure one decimal reduction in D value (5). The thermal resistances of the test organisms were completely characterized by determining the D value at a specific temperature and the z value. The thermal resistance curve of *P. viscosa* is illustrated by Figure 2 and is representative of those obtained for the two other organisms. The calculated D

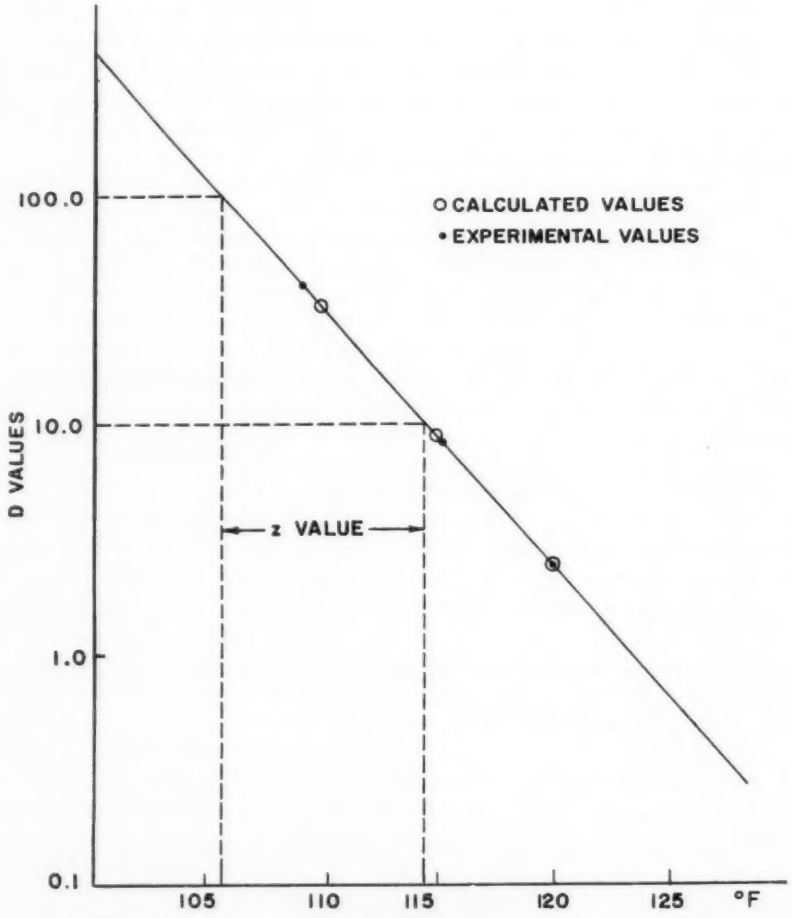

FIG. 2. Thermal resistance curve for *Pseudomonas viscosa* and graphical representation of the z value.

values at 110, 115, and 120° F. and the z values for the three test organisms are given in Table 1.

TABLE 1
Summary of calculated D and z values for three test organisms at three temperatures

Organism	D values			z value
	for 110° F.	for 115° F.	for 120° F.	
Pseudomonas fragi	56.23	22.39	8.91	12.4
Pseudomonas viscosa	30.6	8.4	2.31	8.8
Pseudomonas fluorescens	47.52	11.22	2.64	8.0

In case of *P. viscosa* the thermal destruction rate was observed at 109.2, 115.1, and 120.0° F. The D values calculated were 38.8, 7.85, and 2.36, respectively, for the three temperatures. For *P. fluorescens* the thermal destruction rate was observed at 108.7, 114, and 120° F. The D values obtained were 66.7, 15.2, and 2.63, respectively, for the above temperatures. The thermal destruction rates for *P. fragi* were observed at 109.6, 114.4, and 120.2° F. The D values obtained were 62.5, 21.9, and 9.44, respectively, for those temperatures.

That the survival rates of the three species of psychrophilic organisms were different may be observed from the data presented in Table 1. *P. fragi* demonstrated more resistance to heat than did *P. viscosa* or *P. fluorescens*. These data also show that neither of the organisms should survive the normal pasteurization of milk or skimmilk. The z value for *P. viscosa* obtained in this study is different from the one obtained by Kaufman and Andrews (12) who, of course, used a different strain. These investigators found it to be 14.3. Perhaps because of the difference in growth temperature of the culture, age of culture, or some other variation in experimental technique, Kaufman and Andrews (12) obtained a marked initial lag phase prior to destruction. As can be seen from Figure 1, no lag phase was observed in determining the thermal destruction rate of *P. viscosa* in this study.

Survival of the three test organisms during the cooking process of Cottage Cheese. The data obtained in the preceding section on thermal resistance of the *Pseudomonas* test organisms indicate that these organisms would not survive the cooking period in Cottage Cheese, even though only 120° F. for 30 min. was used. Moreover, since the previous thermal resistance observations were made in sterilized skimmilk at pH 6.4, it was thought that the acidity of the whey at pH 4.7 to 4.5 would likely decrease the survival rate of the organisms. Four lots of Cottage Cheese were made to secure quantitative experimental data on the survival of *Pseudomonas* organisms during the cooking process, where they would be subjected to both heat and acid. Data from the results of these tests are recorded in Table 2. Inspection of the data reveals a low thermal resistance, in the presence of acid, of these organisms. *P. fragi* survived heating to 110° F., as demonstrated by recovery from the curd when the short-time set was used. But, in the long-time set, this organism was destroyed at an even lower temperature, for a zero count was obtained on the

TABLE 2

Number of *Pseudomonas* organisms surviving at different stages of manufacturing Cottage Cheese

No. of samples [a]	Numbers/gram cheese			
	Long-time set	Short-time set		
	Pseudomonas fragi	*Pseudomonas fragi*	*Pseudomonas fluorescens*	*Pseudomonas viscosa*
0	520,000	2,300,000	9,950,000	1,200,000
1a	1,210,000			
1	31,000	133,000	60,000	14,000
2	0	144,000	0	13,000
3	0	50	30	20
4	0	9,500	0	0
5	0	10	20	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	Not taken	Not taken
11	0	0	Not taken	Not taken
12	0	0	0	0
13	0	0	0	0
14	0	0	0	0

[a] Identification of samples: 0—Skimmilk from vat after adding culture of *Pseudomonas;* 1a—Skimmilk 5 hr. after adding culture of *P. fragi;* 1—Before cutting curd at pH 4.7; 2—Curd after heating to 105° F.; 3—Whey after heating to 105° F.; 4—Curd after heating to 110° F.; 5—Whey after heating to 110° F.; 6—Curd after heating to 120° F.; 7—Whey after heating to 120° F.; 8—Curd after heating to 120° F. for 30 min.; 9—Whey after heating to 120° F. for 30 min.; 10—Curd after heating to 130° F. for 30 min.; 11—Whey after heating to 130° F. for 30 min.; 12—Curd after washing and draining; 13—Dressing before use; 14—Finished cheese.

samples of both curd and whey taken at 105° F. In no batch were any of the added psychrophiles able to be recovered from the cheese curd by plating when the temperature had reached 120° F.

One important observation made during the investigation, which was not completely anticipated, was the marked sensitivity of these organisms to developed acidity. Although an incubation temperature of 70 to 86° F. was suitable for the growth of these *Pseudomonas* strains, the number of these organisms per milliliter at the time of cutting the curd for each lot of cheese was less than 10% of the original inoculum in the skimmilk. By the time the pH of the curd had reached 4.7, with no increase in temperature, a 90% reduction of added psychrophiles had occurred. The data in Table 2 demonstrate that the longer exposure to acid development during the long-time set produced greater lethality than did the same acidity under the conditions of the short-time set. The data in Table 2 also show that one generation had probably occurred during a 5-hr. incubation period at 70° F., but 7 hr. later the count had been reduced to 2% of the count at 5 hr.

Other investigators (2, 3), using a different approach to the problem, have also found that a low pH in Cottage Cheese was a critical factor in the growth of psychrophiles.

Enumeration of psychrophiles during storage of Cottage Cheese samples at 38, 50, and 60° F. Samples of Cottage Cheese were plated for psychrophiles

not only during the stages of manufacture, but lots of the creamed cheese were also stored at three different temperatures for definite periods of time and then plated for psychrophilic organisms. Each of the three test organisms developed colonies on P.C. medium which demonstrated recognizable physical differences in shape, size, and color. These characteristics were sufficiently different to be used for purposes of tentative identification. No lot of the stored Cottage Cheese samples showed growth of any of the test organisms which had been used to inoculate the skimmilk previous to making the cheese. It is admitted that the above method of identification is not specific; nevertheless, it is with confidence that the psychrophilic counts, as presented in Table 3, represent a

TABLE 3

Effect of storage on the growth of psychrophilic organisms in Cottage Cheese made from skimmilk inoculated with *Pseudomonas viscosa*

Temperature ° F.	Time	Psychrophilic [a] count/gram of cheese
	1-wk. storage	
38		0
50		2,600,000
60		18,200,000
	2-wk. storage	
38		185,000
50		5,400,000
60		3,600,000
38	4-wk. storage	930,000
38	5-wk. storage	3,200,000

[a] The psychrophilic organisms recovered were not *P. viscosa*.

heterogeneous mixture of contaminants rather than the added organisms, since none of the contaminants exhibited any of the physical characteristics of the test organisms. Data in Table 3 are only for the lot to which *P. viscosa* was added, but nevertheless are representative of the other lots.

The bacterial counts shown in Table 3 clearly indicate that storage temperature was the main factor in controlling the growth of psychrophiles in the creamed cheese, since all lots had essentially the same pH. Zero counts were obtained on samples stored for 1 wk. at 38° F., but after 4 wk. at this temperature the counts were in the range of 300,000 to 900,000 per gram of cheese. However, when the same cheese was stored at 50° F., the psychrophilic count at 1 wk. was approximately 2,000,000 per gram of cheese. After 2 wk. at 50° F., the counts had increased to 5,000,000 per gram. The critical effect of temperature in controlling growth of psychrophiles is in accord with the observations of other investigators (2, 3, 7).

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AVAILABILITY AND METABOLISM OF VARIOUS SUBSTRATES IN RUMINANTS. III. THE EFFECT OF PROPIONATE ON ACETATE OXIDATION IN VIVO

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SUMMARY

An aqueous solution of the sodium salts of acetic acid and acetic acid–1-C^{14} were infused with and without sodium propionate into the jugular vein of a mature dairy steer for periods of 7 hr.

The expired gases were metered, sampled, and analyzed for CO_2 content and its radioactivity determined. From the specific activity of the expired CO_2 and the total CO_2 output, the amount of the infused acetate oxidized was determined. It was shown that propionate fails to inhibit acetate oxidation as drastically as has been observed in vitro. The infusion of propionate with acetate caused a 23.5% reduction in the total volume of CO_2 expired as compared to the infusion of acetate alone. Although the rate of acetate oxidation was slightly depressed and the CO_2 output markedly reduced by propionate, the percentage of the expired CO_2 derived from infused acetate was the same for acetate and acetate plus propionate (21.38 and 21.65%, respectively).

A number of reports have recently appeared in the literature describing propionate as a severe inhibitor of acetate metabolism in animal tissues (3, 4, 6). Although the extent of inhibition appears to vary with the kind of tissue and the species of animals from which it is obtained, the observed effect is always the same, e.g., a marked reduction in acetate oxidation and incorporation into fatty acids. The metabolic step involved in the inhibition has not been established; however, it has been postulated as occurring in the initial activation step of acetate (5).

Since ruminant tissue, especially the liver, is subjected to rather high concentrations of mixtures of acetic and propionic acid, it was thought desirable to study the effect of propionate on acetate metabolism in the intact animal. It is only in the intact animal that the significance of isolated reactions which have been observed in vitro can be ascertained. Therefore, experiments were initiated to study the effect of propionate on the oxidation of acetate-1-C^{14} to carbon dioxide, using a mature dairy steer as the experimental animal.

METHODS AND MATERIALS

Ten days prior to conducting the experiments reported herein, a self-retaining cannula (2) was placed in the trachea of a 340-kg. steer to facilitate the collection and metering of expired gases. Following a 24-hr. fast, one external jugular vein was catheterized according to the method of Ralston *et al.* (7) and the infusion was started. The metabolites were infused into the blood via the catheter with the aid of a constant infusion pump.

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The experimental plan is set forth in Table 1. The acetate solutions were prepared by dissolving Na-acetate 1-C^{14} and nonlabeled reagent grade Na-acetate in distilled water. When propionate was infused simultaneously with acetate, a previously prepared solution of Na-propionate (propionic acid neutralized with NaOH to pH of 7.0) was mixed with the acetate solution. All infused solutions were maintained at a constant temperature (39°) during the course of the experiments with the aid of a thermostatically controlled water bath.

Nelson's method (4) was used to determine glucose in filtrates of blood samples taken at the beginning and end of each experiment. Blood volatile fatty acids were determined chromatographically as described previously (2).

TABLE 1

Experimental plan

Expt. No.	Substrates infused			
	Acetic Acid [a]	Propionic Acid [a]	Acetate-1-C^{14}	Solution infused/hr
	(g/hr)		*(μc/hr)*	*(ml.)*
I	32.74	0	48.24	113.0
II	29.80	24.60	56.32	148.2
III	32.75	24.60	42.09	148.2
IV	29.94	24.60	68.02	148.2
V	32.75	24.60	36.46	148.2

[a] The acids were infused as their sodium salts at rates to supply the amounts of free acids given in the table.

Expired gases were sampled continuously and aliquots removed at 30-min. intervals for 7 hr. The gas samples were assayed for carbon dioxide and radioactivity, as previously reported (2).

RESULTS AND DISCUSSION

One of the primary inhibitory effects of propionate on in vitro acetate metabolism is a pronounced reduction in the conversion of acetate-1-C^{14} to carbon dioxide. If propionate exerts such an effect in vivo, the specific activity of the expired carbon dioxide should be indicative of the extent of the inhibition. As can be seen from Figure 1, the relative specific activity of the expired carbon dioxide was essentially the same for acetate plus propionate as it was for acetate alone. The fact that these curves do not differ suggests that acetate-1-C^{14} supplied an equal fraction of the respiratory CO_2, regardless of the presence or absence of propionate.

To interpret these data without regard to the total volume of carbon dioxide expired would lead to erroneous conclusions concerning the effect of propionate on the metabolism of acetate and, in particular, the general metabolism of the animal. As was seen in Figure 1, the relative specific activity of the expired carbon dioxide was essentially the same for all treatments; however, it can be observed from Table 2 that a smaller percentage of the infused acetate-1-C^{14} was recovered as carbon dioxide when propionate was administered with acetate. These values were calculated using the following equation:

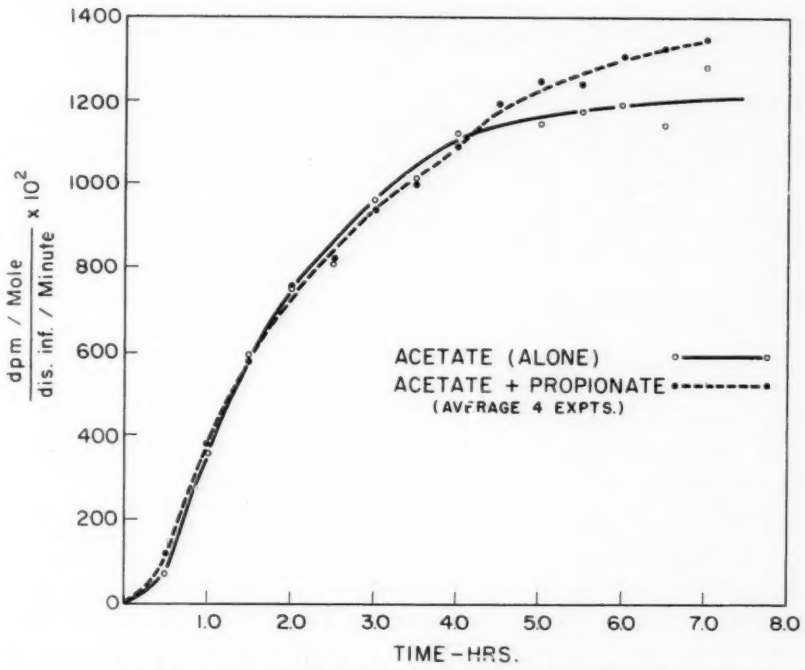

FIG. 1. Effect of added propionate on the relative specific activity of expired CO_2 during the intravenous infusion into a steer of acetate-1-C^{14}.

$$\text{\% of the infused dose recovered in the expired } CO_2 = \frac{\mu c. \text{ expired (sp. ac. of } CO_2 \times \text{total volume of } CO_2) \times 100}{\mu c. \text{ infused as acetate-1-}C^{14}}$$

It follows that the difference in recovery of the infused dose in the expired carbon dioxide was due primarily to differences in the total volume output of carbon dioxide. The data presented in Table 3 show that there was a reduction in CO_2 output when propionate was administered with acetate. When propionate was infused the average hourly decrease in CO_2 output was 799 m*M*, or 23.5% lower, than when acetate was administered alone. The reduced CO_2 output observed during the acetate-propionate experiments can not be fully accounted for by the excess alkali administered in the form of Na-propionate. This amount of sodium (256 m*M*/hr), if retained, would account for only 256 m*M* of CO_2, or roughly one-third of the reduction in CO_2 output (799 m*M*/hr). The fact that starvation alone resulted in values similar to those obtained during the infusion of propionate suggests that acetate alone stimulates carbon dioxide output and this effect is lost when propionate was being metabolized simultaneously. These observations are in agreement with the findings of Armstrong and Blaxter (1), in which the infusion of acetic acid alone resulted in extremely high heat losses associated with its metabolism. The administra-

TABLE 2
Recovery of infused acetate-1-C^{14} in expired CO_2

Time	Acetate alone	Acetate + propionate				
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Mean
(hr.)				*(%)*		
.5	3.61	6.68	5.70	4.00	4.77	5.29
1.0	10.99	13.14	14.78	9.51	9.57	11.75
1.5	17.12	20.89	16.44	11.66	14.63	15.91
2.0	24.13	28.21	19.30	15.63	19.30	20.61
2.5	27.93	31.35	21.46	18.55	22.87	23.56
3.0	32.79	34.05	23.72	21.80	25.98	26.39
3.5	36.50	36.74	25.35	24.02	28.34	28.61
4.0	39.46	38.58	27.54	25.97	31.50	30.90
4.5	43.72	41.58	29.64	27.94	34.25	33.35
5.0	46.12	43.82	31.35	29.83	36.53	35.38
5.5	47.66	45.67	33.02	31.53	38.21	37.11
6.0	49.59	47.80	34.92	33.32	40.32	39.09
6.5	50.38	49.37	36.43	34.61	.....	40.13
7.0	52.07	50.76	37.98	35.68	.....	41.47

tion of a mixture of the volatile fatty acids (acetic, propionic, and butyric) resulted in a lower heat increment and, henceforth, more efficient utilization of the acids. The metabolic step, or the particular tissue whereby acetic acid exerts its high specific dynamic action, has not been established. The data presented here suggest that the site of this effect is the liver. In defense of this postulate one can point to the following facts: (a) Propionate is almost quantitatively removed by the liver and, therefore, is not present in other tissues. The infusion of 24.6 g/hr of propionate failed to raise the peripheral blood level to measurable amounts. (b) If propionate was metabolized only by the liver, then the effect it exerted on acetate metabolism must have been at this site. More information is needed to substantiate or refute this postulate.

The reduced metabolism during propionate infusion as indicated by the lower CO_2 output resulted in a lower acetate oxidation rate. These data are presented in Table 4. The acetate oxidation rates observed from the third through the seventh hours are considered to be the true oxidation rates under these conditions. The data obtained for the oxidation rate of acetate, when acetate alone was infused, are in excellent agreement with data reported earlier

TABLE 3
Effect of acetate and acetate plus propionate infusions on the amount of carbon dioxide expired

Time	Acetate alone	Acetate + propionate				None [a]
	I	II	III	IV	V	
(hr.)			*(mM)*			
3–4	3,341	2,715	2,571	2,395	2,460	2,730
4–5	3,668	2,752	2,585	2,491	2,553	2,749
5–6	3,348	2,600	2,633	2,543	2,896	2,513
6–7	3,236	2,644	2,597	2,544	.....	2,715
Mean	3,398	2,678	2,590	2,493	2,636	2,677

[a] Animal fasted 24 hr.—no substrate.

TABLE 4
Effect of propionate on the amount of acetate oxidized to carbon dioxide

Time	Acetate alone	Acetate + propionate				
	I	II	III	IV	V	Mean
(hr.)			*(g.)*			
3–4	19.47	15.54	12.78	11.52	15.74	13.90
4–5	23.82	19.30	15.26	13.56	18.54	16.66
5–6	21.92	19.64	17.29	15.20	19.41	17.86
6–7	21.92	20.96	18.48	14.92	.....	18.12
Mean	21.78	18.86	15.95	13.80	17.90	16.64

(2). Even though the oxidation of acetate was somewhat less when propionate was infused simultaneously, it was noted that there was no difference in blood levels of acetate (approximately 5.0 mg.% in all experiments). This suggests that propionate stimulates anabolic reactions involving acetate.

The percentages of the expired carbon dioxide derived from the oxidation of infused acetate are given in Table 5. Even though the administration of propionate reduced both the oxidation of acetate and, in particular, the quantity of carbon dioxide expired, the same proportion of expired CO_2 was derived from infused acetate, whether infused alone or with propionate.

TABLE 5
Effect of propionate on the amount of carbon dioxide derived from infused acetate

Time	Acetate alone	Acetate + propionate				
	I	II	III	IV	V	Mean
(hr.)			*(%)*			
3–4	19.43	19.08	16.57	16.03	19.27	17.74
4–5	21.65	23.36	19.65	18.15	22.48	20.91
5–6	21.83	25.19	21.88	19.90	25.75	23.18
6–7	23.59	33.35	23.72	19.54	.....	25.54
Mean	21.38	25.25	20.46	18.41	22.50	21.65

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PRODUCTION AND ABSORPTION OF VOLATILE FATTY ACIDS IN THE PERFUSED RUMEN

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SUMMARY

The rumens from three mature goats were perfused with homologous heparinized blood to measure the rate of volatile fatty acid (VFA) production and absorption. An effort was made to maintain an active microbial population and fermentation during the perfusion by approximating normal conditions with respect to anaerobiosis, temperature, substrate, and removing the end products of fermentation.

Volatile fatty acid absorption rates were much below expected values, which may be attributed to a slow rate of blood flow. The average blood flow rate for the three perfusions was 17 ml/min. The inability to remove the fatty acids from the rumen via absorption may have affected the production rates which were: acetic, 2.61 g/hr; propionic, 1.16 g/hr; and butyric, 0.89 g/hr. In contrast to reports in the literature, the relative proportions of VFA's in the rumen vein blood (A,51; P,26; B,23) were nearly identical to those in the rumen fluid (A,55; P,22; B,23). Glucose utilization by rumen tissue was demonstrated by arteriovenous differences of 6-10 mg.%. Low levels of ketone bodies were observed in venous blood.

Three other perfusions were conducted to study the effect of radical changes in the proportions of the VFA's in the rumen on the distribution of these acids in the perfusate. The addition of either propionic or butyric acids, which shifted the ratio of the acids in the rumen field, caused drastic changes in proportions of these acids in the rumen vein blood. Although blood VFA ratios did not show a linear relationship to the ratios observed in the rumen fluid, it was apparent that the acids were absorbed at rates depending on their concentration in the rumen. The addition of butyric acid to the perfused rumen brought about increases in ketone body levels in the venous blood. Ketone body production by the rumen tissue may be of importance under conditions where high levels of butyric acid are present in the rumen.

Quantitative measurements of volatile fatty acid (VFA) production in the rumen are essential to the definition of energy metabolism in ruminants. That these acids are the principal products of microbial fermentation in the rumen has been clearly established (2, 3). It has been further demonstrated that considerable quantities of the volatile acids enter the circulation via absorption through the rumen wall (1, 2, 4, 7). The extent of the metabolic contributions of the individual acids is not known because quantitative determinations of the rumen fermentation products are not reliable. Thus, Emery *et al.* (5) have estimated from turnover curves based on changes in rumen volatile acid concentration with time after feeding that rumen volatile acid production in steers would supply from 3 to 13% of the total energy requirement, whereas Carroll and Hungate (3) have estimated from in vitro fermentation rates that approximately 70% of a steer's energy requirement could be furnished by the metabolism of the volatile fatty acids produced in the rumen. The in vivo volatile acid absorption data of Pfander and Phillipson

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(13) also show the total volatile acid contribution to be about 70% of the resting requirement, although the data for individual acids vary considerably from those of Carroll and Hungate.

Because most of the volatile acids produced in the rumen are apparently absorbed through the rumen wall, measurements of the volatile acids entering the rumen vascular circulation during a 24-hr. period would provide the most accurate estimate of the metabolic contribution of the rumen volatile acids. Direct measurements under normal physiological conditions in vivo are difficult if not impossible. Accurate estimates could be made if the absorptive process for each of the acids could be quantitatively defined in terms of more easily measured criteria, such as rumen acid concentration, rumen pH, and rate of blood flow through the rumen vascular system. Perfusion of the isolated rumen is a technique which permits precise simultaneous measurements of these criteria as well as the rate of volatile acid absorption.

McCarthy *et al.* (9) of the Maryland Station reported the results of rumen perfusion studies concerning the production and absorption of volatile fatty acids. They concluded that "the rumen perfusion technique is an excellent method for estimating the production of rumen volatile acids." In this paper are presented the results of perfusion studies at this laboratory on the production and absorption of rumen volatile acids.

EXPERIMENTAL PROCEDURE

Materials. The rumina from mature female goats were used for the perfusions reported in this study. Each goat was fasted for 24 hr. and then offered 2 lb. of alfalfa hay and 1 lb. of a 14% protein concentrate mixture 1 to 2 hr. prior to slaughter. The goats were anesthetized with pentobarbital sodium (Nembutal Sodium, Abbott) and exsanguinated via a cannula inserted into the carotid artery. The blood was collected in a two-liter flask containing heparin. As soon as blood had ceased to flow from the carotid cannula, the abdominal cavity was opened from the left side. Ligatures were tied around the esophagus adjacent to the cardia and around the duodenum immediately posterior to the pyloric sphincter. The entire stomach was freed from adhering tissue and removed from the body. Particular care was taken to minimize damage to the right ruminal vessels where they leave the surface of the anterior dorsal sac of the rumen.

Glass cannulas were inserted into the right ruminal artery and vein and the stomach was placed in a 0.9% saline bath at a temperature of 39° C. The arterial cannula was connected by tygon tubing to a gravity flow bottle suspended approximately 5 ft. above the surface of the saline bath. A tygon tube connected to the venous cannula terminated in a 500-ml. graduated cylinder.

Approximately 500 ml. of physiological saline was permitted to flow through the rumen vascular system to flush out residual blood. Samples of the saline perfusate were saved for VFA analysis. Following the saline rinse, samples of rumen fluid and arterial blood were obtained and the perfusion was initiated with the previously collected heparinized blood that had been filtered through

glass wool and cotton. Usually, sufficient blood was collected during exsanguination to allow 3 or 4 hr. of perfusion without recirculating the blood. In one instance, venous blood was recirculated without oxygenation. The results of this study are discussed below.

Rumen fluid samples were obtained by means of a tygon tube inserted into the rumen through the esophagus. The samples were collected in a suction flask under slightly reduced pressure. Arterial blood samples were obtained from the gravity flow bottle at the beginning and end of the experiment. Venous blood samples were obtained at intervals during the perfusion from the graduated cylinder. Blood flow rates were measured as the volume of venous blood collected during a specified time. With careful cannulation, it has been possible to perfuse the isolated rumen without any leakage of blood from the vascular system.

Chemical. Volatile fatty acids were determined chromatographically by a modification of the method of Neish (10). Rumen samples were acidified to pH 2.0 with 10N H_2SO_4 and 1-ml. aliquots were mixed with celite and packed on top of a previously prepared column. Volatile fatty acid determinations in blood were made on the protein-free filtrate from 20-ml. samples. The tungstic acid precipitate was washed several times with distilled water. The filtrate and washings were made alkaline with NaOH and reduced to a volume of 2 to 3 ml. under reduced pressure at 60° C. The concentrated filtrate was acidified with H_2SO_4, mixed with 2 to 3 g. of celite, and packed on the top of a chromatographic column.

Blood glucose was estimated by the reducing sugar method of Somogyi (14), as modified by Nelson (11). Blood ketones were determined as acetone by the method of Greenberg and Lester (6).

RESULTS AND DISCUSSION

Perfusions 1, 2, and 3 were conducted to determine the rate of volatile fatty acid production and absorption in goat rumens under conditions approximating normal. Feeding the animals prior to slaughter provided normal substrate for an active fermentation during the experimental period.

The average body weight for the three goats prior to slaughter was 133 lb. The initial fluid volume of the rumen was estimated from the weight and moisture content of the rumen ingesta at the end of the perfusion, after correcting for the volume of rumen samples taken during the course of the experiment.

The initial rumen sample was obtained immediately following the saline rinse; hence, the changes in concentration of VFA's during the perfusion with saline were not included in the calculation of VFA production. However, it is interesting to note (Table 1) that the fatty acids were absorbed into the saline at fairly high concentrations. Also, the relative distribution of the acids in the saline perfusate was similar to the distribution in the initial rumen fluid samples.

One of the difficulties encountered in perfusion studies is that of obtaining physiological blood flow rates through the vascular systems of the perfused

TABLE 1
Concentration and distribution of VFA in the saline perfusate and rumen fluid

Perfusion No.		VFA concentration	Distribution *(molar %)*		
			Acetic	Propionic	Butyric
		(mg.%)	*(%)*		
1	Saline	28.4	50.4	27.2	22.4
	Rumen fluid	689.8	53.0	23.3	23.7
2	Saline	17.5	56.6	22.9	20.5
	Rumen fluid	769.0	58.2	18.8	23.0
3	Saline	14.5	52.6	20.7	26.7
	Rumen fluid	728.8	54.6	21.0	24.4

TABLE 2
Blood flow rate through the perfused rumen

Perfusate	Time interval	Total collected	Rate
	(min.)	*(ml.)*	*(ml/min)*
Saline	11	740	67.3
Arterial Blood	30	640	21.3
Arterial Blood	30	485	16.2
Arterial Blood	30	620	20.9
Venous Blood	12	790	65.8
Venous Blood	6	570	95.0
Venous Blood	6	420	70.0

organ. As can be observed from the blood flow data presented in Table 2, the perfusion of the rumen was no exception. These data were obtained during Perfusion 3, when venous blood was recirculated without oxygenation following the usual experimental period. Saline flowed at a relatively rapid rate as did venous blood, whereas a marked depression in flow rate occurred when the perfusate was arterial blood. This is in agreement with the results obtained by Kumar *et al.* (8), who reported that the hyperoxygenated blood flowed more slowly than did venous blood in the perfused mammary gland. Although venous blood flowed at a more desirable rate, there were no volatile fatty acids absorbed and there was no uptake of glucose during passage through the rumen.

The total VFA production was calculated from the increase in VFA content of the rumen ingesta during the experimental period and the VFA's absorbed into the perfused blood. The mean values for the three perfusions

TABLE 3
Rate of VFA production in the perfused rumen

VFA	+ △ VFA during perfusion				Rate
	Rumen	Blood	Total (+B)		
	(mg.)			*(% dist.)*	*(mg/min)*
C_2	5,410	241	5,651	56.0	43.5
C_3	2,390	124	2,514	24.9	19.3
C_4	1,810	110	1,920	19.1	14.8
Σ	9,610	474	10,085	100.0	77.6

are presented in Table 3. The total VFA production for the perfusions averaged 10 g. or 77.6 mg/min. It is of interest that the volatile fatty acids produced during the perfusion period were in the same relative proportion as the VFA's present at the beginning of the experiment. The values of 56.0, 24.9, and 19.1% for acetic, propionic, and butyric acids compare with values of 54.5, 21.3, and 24.2%, respectively, for the acids in the initial rumen fluid sample.

The absorption data (Table 4), while reflecting a low rate of absorption

TABLE 4
Absorption of VFA from the perfused rumen

VFA	Blood VFA				Rate
	Initial		Net increase		
	(mg.%)	*(% dist.)*	*(mg.)*	*(% dist.)*	*(mg/min)*
C_2	5.99	88.7	241	50.8	2.20
C_3	0.00	0.0	124	26.2	1.14
C_4	0.77	11.3	110	23.0	1.00
Σ	6.76	100	474	100	4.34

into the blood, show a marked similarity in relative proportions of the volatile fatty acids to the distribution occurring in the rumen fluid. Of particular interest is the absorption of 110 mg. of butyric acid when the total VFA uptake was only 474 mg. These data substantiate the results of rumen perfusion studies reported by McCarthy *et al.* (9). The low butyric acid values reported by others (1, 4) for rumen vein blood or portal blood have been attributed to the conversion of butyric acid to β-hydroxy-butyric acid by rumen tissue, as suggested by the work of Pennington (12). However, only a small increase in ketone body concentration (average of three experiments 1.79 mg.%) was noted during these experiments. Glucose uptake as measured by the arteriovenous difference in the concentration of total reducing substances averaged 11.3 mg.%. This is regarded as providing evidence for the integrity of the rumen tissue during the perfusions.

The data presented in the preceding pages on the absorption of the volatile fatty acids must be interpreted in light of the extremely low blood flow rates through the vascular system of the perfused rumen. Since the flow rates were unphysiological, one must conclude that the absorption rates were also abnormal. However, it is felt that the relative proportions of the acids in the perfusate is typical of what might be expected in the rumen vein of the intact animal. The data presented show that the relative proportion of the volatile acids in the venous blood compares closely with the acid distribution found in the rumen ingesta, suggesting that the acids were absorbed in direct proportion to their concentration in the rumen. Due to the constancy of the proportions of the VFA's found in the rumen fluid under the experimental conditions imposed, no information was available as to the effect of changes in the VFA distribution on the relative proportions of the absorbed acids in the perfusate. Therefore, three perfusions were conducted to observe the effect of radical changes

in the proportions of the VFA's in the rumen on the distribution of these acids in the blood.

The experimental procedure was the same as that followed in the first three perfusions, except that the VFA ratios were altered by the addition of either propionic acid (14.4 g.) or butyric acid (13.6 and 9.6 g., respectively, for Experiments 1 and 2) after a perfusion period of from 30 to 60 min. to establish the normal distribution in both rumen fluid and perfusate. The perfusions were continued for an additional 45- to 60-min. period to measure the effect of the added acids. The pH of the rumen fluid remained within the normal range throughout all perfusions, and protozoal activity appeared unaltered.

The effect of the addition of propionic acid to the perfused rumen on the VFA distribution in the rumen fluid and perfusate (blood) is shown in Table 5.

TABLE 5

Effect of the addition of propionic acid to the perfused rumen on the distribution of VFA in the perfusate

	Rumen fluid			Blood		
Sample No.	0	1 [a]	2 [b]	0	1 [a]	2 [b]
VFA content *(mg.%)*	480	516	1,232	6.5	35.7	47.9
Distribution						
Acetic *(%)*	54.0	56.0	46.0	75.3	56.0	36.7
Propionic *(%)*	12.0	18.0	36.0	4.6	20.2	49.1
Butyric *(%)*	34.0	26.0	18.0	20.1	23.8	14.2

[a] Sample taken 1 hr. after perfusion started, but prior to propionate addition.
[b] Sample taken 1 hr. after proponiate was added.

The administration of propionic acid shifted the proportion of this acid in the rumen fluid from 18.0% of the total VFA's to 36.9%. This change in the proportion of propionate was reflected dramatically in the blood (Sample 1 versus Sample 2). Although the response observed in the change of propionate in rumen fluid as compared to blood was not linear, it does substantiate the findings in the three normal perfusions, e.g., the volatile acids are absorbed from the rumen in proportion to their concentration.

The data obtained during the first butyric acid experiment are given in Table 6. Here again the change in the relative proportions of the VFA's in the rumen fluid is reflected in the venous blood (Sample 1 versus Sample 2).

TABLE 6

Effect of the addition of butyric acid to the perfused rumen on the distribution of VFA in the perfusate (Butyrate Expt. 1)

	Rumen fluid			Blood		
Sample No.	0	1 [a]	2 [b]	0	1	2
VFA content *(mg.%)*	394	400	924	4.8	24.2	30.7
Distribution						
Acetic *(%)*	50.0	53.0	26.0	74.7	60.7	46.9
Propionic *(%)*	12.9	16.3	8.2	00.0	20.2	15.5
Butyric *(%)*	37.1	30.8	65.5	25.3	19.1	37.6

[a] Sample taken 50 min. after perfusion, started just prior to butyrate addition.
[b] Sample taken 1 hr. after butyrate addition.

The fact that the shift in the proportion of butyric acid was not as great in the blood as in the rumen fluid may be explained partially by the differences in time that the samples were taken. Rumen fluid Samples 1 and 2 were taken just prior to the addition of the acid and at the end of the experiment, respectively; whereas, Blood Samples 1 and 2 represent the mean of two samples taken prior to and following the acid administration, respectively. Therefore, the blood samples might be expected to show less change than the rumen fluid samples.

The second butyrate experiment shows essentially the same picture with respect to changes in butyric acid percentages in the rumen fluid and blood as did the first experiment (Table 7). There was an apparent conversion of

TABLE 7

Effect of the addition of butyric acid to the perfused rumen on the distribution of VFA in the perfusate (Butyrate Expt. 2)

	Rumen fluid			Blood		
Sample No.	0	1[a]	2[b]	0	1	2
VFA content *(mg.%)*	563	593	1,291	54	28.3	44.1
Distribution						
Acetic *(%)*	59.7	59.9	41.4	90.7	60.4	51.0
Propionic *(%)*	25.0	23.8	16.1	9.3	20.9	22.4
Butyric *(%)*	15.3	16.3	42.5	0.0	18.7	26.6

[a] Sample taken 30 min. after perfusion began, just prior to butyric addition.
[b] Sample taken 1 hr. after butyrate addition.

butyrate to acetate and propionate because the increase in acetate and to a lesser extent propionate production after butyrate addition was greater than that observed during the first hour prior to butyrate addition. This observation is in agreement with the tracer data of McCarthy *et al.* (9).

When the concentration of butyrate in the rumen was increased by the addition of butyric acid the level of ketones in rumen vein blood increased to 6-8 mg.% as compared to 1-2 mg.% in arterial blood. Therefore, it appears that unusually high levels of butyric acid in the rumen and the greater transfer rate across the rumen wall may be conducive to ketone formation by the rumen tissue. As in the previous experiments, the rumen tissue removed glucose from the arterial blood at a fairly constant rate, which suggests that the integrity of the tissue was maintained.

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EFFECT OF PHLORIDZIN, INSULIN, AND BUTYRATE ON THE CONCENTRATION OF GLUCOSE AND KETONES IN THE BLOOD AND URINE OF FASTED STEERS

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SUMMARY

Seven experiments were conducted in an attempt to produce both clinical and biochemical symptoms of bovine ketosis. The treatments imposed were phloridzin, which causd a lowering of the renal threshold for glucose, phloridzin plus 800 units of insulin, which depressed the total reducing substances (T.R.S.) level in the blood during a period of increased glucose demand due to the lowered renal threshold, and the infusion of 25 g. per hour of butyrate, either by itself or with phloridzin and with phloridzin and insulin.

When phloridzin was infused following a 48- to 72-hr. fast, 56.62 g. of glucose were voided in the urine in 8 hr. Only a moderate fall in blood T.R.S. level and a moderate rise in blood and urine ketone levels were observed.

When two intramuscular injections of 400 units of insulin were superimposed on phloridzin infusion, the blood T.R.S. level rapidly dropped to the ketotic level. The blood and urine ketone levels were less than with phloridzin alone and less glucose was voided in the urine.

The infusion of butyrate at the rate of 25 g. per hour caused a very rapid drop in the T.R.S. level of the blood. Even at this high level of butyrate infusion, the level of ketones found in the blood approached ketotic levels only if phloridzin was infused concurrently. This observation adds support to the observation and theoretical postulation that a glucose stress is a predisposing factor in ketone formation. The butyrate concentration in the blood increased to a peak early in the infusion period, followed by a reduction and the establishment of a plateau. Approximately 185 g. of butyrate were thus metabolized during an 8-hr. period without adverse effect upon the animal.

No clinical symptoms of hypoglycemic shock were observed under any of the experimental treatments applied.

One of the primary deterrents to a solution of the cause-and-effect relationships involved in bovine ketosis has been the insidiousness of the disorder and an inability of research workers to produce the syndrome at will. Bovine ketosis is defined as a metabolic disorder of high-producing cows that is characterized by low blood glucose, elevated blood and urinary ketone bodies, poor appetite, loss in milk production, and either lethargy or high excitability. Paresis or incoordination may be present and a rapid loss in condition occurs if the syndrome is not corrected (17). The death loss from bovine ketosis is negligible because spontaneous recovery usually occurs when milk production has been depressed sufficiently. Although few animals die of the disorder, milk production for the entire lactation is considerably reduced as is income from milk.

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[1] The data presented are from a thesis submitted by the senior author to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The currently popular compounds that are used in treating ketotic cows or attempting to prevent its occurrence in cows with a previous history of the upset are, (a) glucose and/or other monosaccharides, (b) propionates, glycerol, or lactates, and (c) adrenocorticotrophic hormone (ACTH), adrenocorticoids, or synthetic compounds with adrenocorticoid activity. These treatments and preventive measures would each add in some manner to the amount of available carbohydrate in the system. The compounds in Group 1 add carbohydrate directly to the blood. The Group 2 compounds are made up of three-carbon units that are glucogenic if absorbed unchanged from the rumen or are converted by rumen bacteria to other three-carbon compounds that are glucogenic when absorbed. The hormones and hormone-like substances in Group 3 step up gluconeogenesis primarily from amino acids by increasing the endogenous production of adrenocorticoids via ACTH, or increasing the corticoid level by adding the hormone or hormone-like substance from an exogenous source.

A high level of milk production is usually the stressing factor postulated as the cause of ketosis in dairy cattle. Because of the curative effect of glucose and glucogenic substances, it is equally plausible and perhaps more precise to postulate that the high requirement for carbohydrate in the synthesis of lactose is the stressing agent. For example, a cow producing 25 kg. of milk containing 4.8% lactose would need 1,200 g. of carbohydrate per day for lactose synthesis alone. This amount is greater than that estimated to be available from the propionic acid formed in the rumen of a cow on full feed (17).

One problem encountered in studies of bovine ketosis has been the lack of a suitable method for experimental production of the disorder. Withholding feed from lactating cows results in an immediate decline in milk production which apparently eliminates the causative stress. If a glucose stress is the immediate cause of ketosis in lactating cows, then it should be possible to produce ketosis in steers, provided that a suitable glucose stress could be imposed. In the studies herein reported, phloridzin was administered to fasted steers to provide a prolonged glucose drain.

EXPERIMENTAL PROCEDURE

A Holstein steer which weighed about 350 lb. was used in all seven experiments. In each case a plastic catheter was placed in the left exterior jugular vein according to the method of Ralston *et al.* (14). The various solutions were infused through the catheter [2] into the blood and blood samples were withdrawn through it. Drawing blood samples in this manner allows the removal of blood without causing discomfort to the steer or exciting him in any way. Between trials that were run on consecutive days, the tube was filled with heparinized 0.9% sodium chloride and sealed at the end with heat. All infusion solutions were administered with an intravenous drip apparatus and a gravity flow blood serum bottle. In the first four experiments the solution was infused as fast

[2] PE 205 Intramedic Polyethylene Tubing (Adams), 16-gauge needle fits into tubing Tubing passes through 12-gauge needle. (i.d. .062 in.—o.d. .082 in.)

as it would flow through a 16-gauge needle through the jugular catheter. In the last three experiments the flow rate was regulated so that one liter of solution was infused each hour.

All of the solutions used were made isotonic with mannitol, which is non-metabolizable by mammalian tissue (7, 10).

Blood proteins were precipitated by the method of Somogyi (18) for both ketone and total reducing substances analyses. Total reducing substances were measured by Nelson's (11) modification of the Somogyi method (18).

Ketones, calculated as acetone, were measured by a modification of the Greenberg and Lester (8) method in which 3 instead of 2 cc. of carbon tetrachloride were used for extraction, in order to provide enough volume for the readings to be made in a Bausch and Lomb Spectronic 20 Colorimeter.

Somogyi filtrates were made on the urine samples for ketone analyses. Urinary glucose was measured in activated charcoal filtrates with Glucostat,[3] a commercially available glucose-oxidase and chromogen preparation. In experiments in which phloridzin was infused, one to 99 dilutions were necessary to obtain color development which was in a suitable range for reading in the colorimeter.

Blood volatile fatty acids were determined by the method described in a previous publication from this laboratory by Davis, Brown, Staubus, and Nelson (4).

Experiments 1 and 3 were run after the steer had been fasted for 48 and 72 hr., respectively. Isotonic mannitol solution (56 g. per liter) containing 0.8 g. of phloridzin per liter was dripped into the jugular as rapidly as possible (approximately 1.54 liter/hour) for an 8-hr. period. Zero time and hourly jugular blood samples were collected in tubes containing heparin and sodium fluoride. These samples were immediately refrigerated at 40° F. and the proteins were precipitated within 4 hr. after completion of the experiment. Individual urinations were collected, measured, and sampled. The urine samples were immediately frozen until analyzed.

Experiment 2 was conducted the day after Experiment 1. Experiment 4 was conducted the day following Experiment 3. No feed was available to the animal between Experiments 1 and 2 or Experiments 3 and 4. In Experiments 2 and 4, 400 units of insulin were administered intramuscularly to the steer at zero time and 4 hr. after the infusion of isotonic mannitol and phloridzin was begun. Samples were taken in the same manner as in the two previous experiments.

Experiment 5 measured the response of a normal steer starved 24 hr. to the infusion of 1 liter per hour of isotonic mannitol containing 25 g. of butyric acid neutralized with sodium hydroxide. Samples were collected and stored in the same manner as in the previous experiments, except that enough blood was withdrawn at 0, 2, 4, 6, and 8 hr. to allow analyses for volatile fatty acids.

Experiment 6 measured the response of the same steer after an additional 24-hr. fast to a butyrate infusion and glucose stress mediated by the addition

[3] Worthington Biochemical Corporation, Freehold, New Jersey.

of 0.8 g. of phloridzin per liter of infusate. Sampling proceeded as in Experiment 5.

In Experiment 7, following an additional 24-hr. fast, two subcutaneous injections of 400 units of insulin were superimposed on the treatment administered in Experiment 6. Samples homologous to those in Experiments 5 and 6 were collected.

EXPERIMENTAL RESULTS

Figure 1 shows the effect of phloridzin and phloridzin plus insulin administration on the concentration of T.R.S. in the blood. After a starvation period of 96 hr., the steer was able to maintain the total reducing substances (T.R.S.) in the jugular blood within the normal range. On the first day of the experiment the mean zero time T.R.S. was 47.4 mg.% and on the following day it

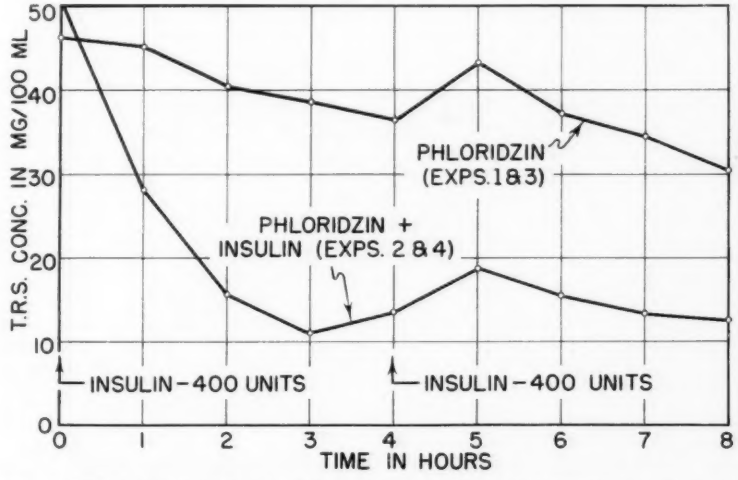

FIG. 1. Effect of phloridzin and phloridzin plus insulin administration on the concentration of total reducing substances in the blood of a steer.

was slightly higher, 50.2 mg.%. During the course of an 8-hr. infusion of isotonic mannitol and phloridzin there was a progressive drop in T.R.S., but even at the end of the period, the animal was still maintaining a level of 30.4 mg.%, which is above the level that is generally considered to be indicative of ketosis. However, under the influence of both phloridzin and 400 units of insulin, the blood T.R.S. level dropped rapidly from 50.2 to 11.0 mg.% at the end of 3 hr. After reaching this low, some recovery occurred at 4 hr. and continued, in spite of the intramuscular injection of 400 additional units of insulin, to the fifth hour, after which a slow decline began. However, it should be noted that from the third hour on, the blood T.R.S. level was within a range that is usually associated with severe ketosis.

The average magnitude of the glucose stress mediated by phloridzin was 55.81 and 30.81 g. in Experiments 1 and 3 and 2 and 4, respectively. It is apparent that the amount of glucose which was filtered out and excreted in the urine was somewhat dependent upon the level present in the blood which passes through the kidney. Although the T.R.S. data cited in Figure 1 are from jugular blood, it is probably indicative of the level present in the arterial blood passing through the kidney in relative if not absolute terms. Therefore, it is not surprising that a greater amount of glucose was voided in the urine when phloridzin was used alone than was voided when insulin injections were superimposed on phloridzin. The experimental glucose drain resulting from phloridzin and phloridzin plus insulin treatment is equal in magnitude to the more normal glucose stress of a 1,200-lb. cow producing 28.76 and 16.28 lb. of milk, respectively, assuming 100% conversion of glucose to lactose by the mammary gland. This production should also be considered to be existing after from three to five days of fasting.

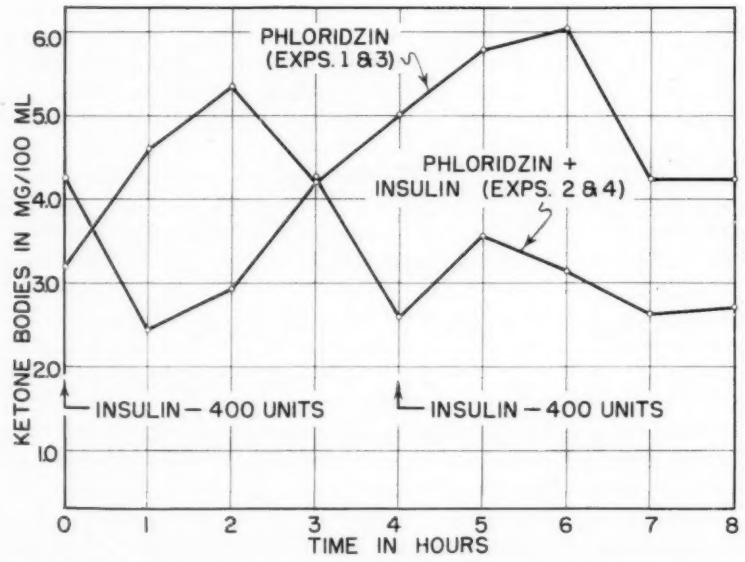

FIG. 2. Effect of phloridzin and phloridzin plus insulin administration on the concentration of ketone bodies in the blood of a steer.

Figure 2 shows the concentration of ketone bodies, measured as acetone, observed in jugular blood throughout Experiments 1 through 4. It is readily apparent that although the concentration of ketone bodies did not exceed normal fasting levels, even with an artificial glucose stress, the injections of insulin, in spite of lowering the blood T.R.S. level, appeared to reduce the buildup of blood ketone bodies.

This difference in buildup of ketone bodies in the jugular vein blood was not due to a differential rate of spillage into the urine, because the cumulative excretion into the urine averaged 554 mg. in Experiments 1 and 3 and 520 mg. in Experiments 2 and 4. Such spillages are insignificant and reflect the lack of ketone body buildup in the blood.

To eliminate the possibility that this rather surprising lack of ketone body formation was due to a lack of ketogenic substances, the steer was placed on a normal ration with a high proportion of grain for a month and then isotonic mannitol and butyric acid, which is highly ketogenic, were infused together, with phloridzin and with phloridzin and subcutaneous injections of insulin.

It is interesting to note in Figure 3 that in all three experiments in which 25 g. of butyric acid were infused per hour the T.R.S. dropped to ketotic levels in 3 hr. or less. In fact, the curve for butyric acid alone seems to drop from its higher initial T.R.S. level at the same rate as the curve for butyric acid plus phloridzin plus insulin. Following the initial drop, the T.R.S. level stabilized between 15 and 20 mg.%.

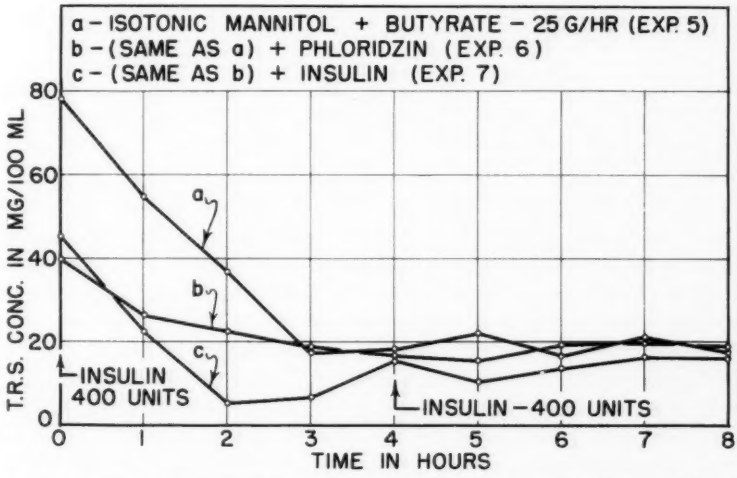

FIG. 3. Effect of butyrate administration with and without phloridzin and insulin on the concentration of total reducing substances in the blood of a steer.

Butyric acid alone caused an insignificant excretion of glucose into the urine. The excretion due to the inclusion of phloridzin in the infusate and insulin injections gave totals of 56.62 and 38.13 g., respectively, as compared with 54.40 and 30.81 g. when no butyrate was administered. Such close agreement is somewhat surprising in light of the lower blood T.R.S. levels during the infusion of butyrate.

The ability of the steer to utilize high levels of ketogenic substances is shown in Figure 4. In spite of the infusion of 25 g. of butyric acid per hour,

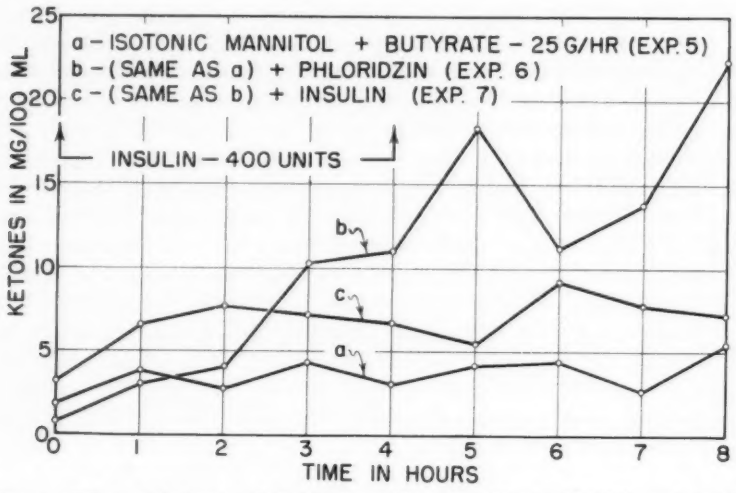

FIG. 4. Effect of butyrate administration with and without phloridzin and insulin on the concentration of ketone bodies in the blood of a steer.

the blood ketone level did not exceed the normal range in the case of butyrate alone or butyrate plus phloridzin plus insulin. Only in the case of butyrate plus phloridzin did the concentration exceed normal. Even then it rose only to the lower limit of that which is considered ketotic.

The cumulative urinary excretion of ketone bodies in Experiments 5, 6, and 7 amounted to 1.76, 3.54, and 3.12 g., respectively. The similarity in the cumulative amounts of ketone bodies excreted in the urine is a bit surprising. Under the current theory of unselective filtration of substances by the glomeruli of the kidney and selective resorption by the tubules, one would expect total excretion to follow the concentration in the blood quite closely. However, under the conditions of these experiments very nearly the same amounts were excreted either with or without insulin and 50% as much was excreted with butyrate alone.

Since such a large amount of butyric acid was infused in each experiment, the concentrations of the volatile fatty acids in jugular blood were measured at 2-hr. intervals. These concentrations are plotted in Figure 5. Propionate was not present in the jugular blood in measurable quantities at any time during the butyrate experiments. The acetate level was very similar in all three tests and approximated normal fasting levels, so the acetate values are plotted as a composite value for all three experiments.

In all three butyrate experiments, there was a considerable increase in the butyrate level of the blood. The zero-time sample in the first two experiments showed a negligible amount which is normal and showed no residual carryover from Experiments 5 to 6. In Experiment 7 there appeared to be some slight carryover, but it amounted to only 1.32 mg.%. However, in all cases, 2 hr. after

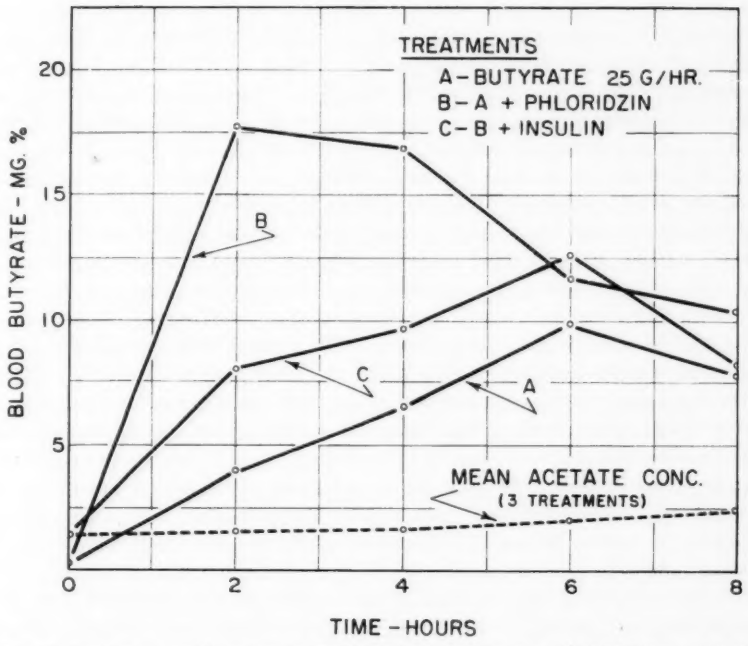

FIG. 5. Butyrate concentration in jugular blood during the administration of butyrate with and without phloridzin and insulin.

the infusion began the concentration of butyrate began to increase. This deviation from normal can be attributed to two possible causes. The first possibility is that 25 g. of butyrate per hour is more than the animal normally would absorb and, thus, the threshold of the liver's ability to remove and metabolize butyrate has been exceeded. The second possibility is that under normal conditions all of the butyrate which is available comes from the rumen and goes directly to the liver; whereas, under the conditions of these experiments butyrate was introduced into the jugular vein and thus was mixed throughout the circulating blood before the liver had an opportunity to metabolize it. However, such an explanation is not consistent with the decrease in concentration observed during the last 2 hr. while the rate of infusion remained constant. Such a decrease is to be expected only under conditions of increased rate of metabolism. Why such a response should occur and how it is mediated is not clear.

DISCUSSION

The observations made in these seven experiments indicate that the bovine's ability to maintain homeostasis or nearly normal physiological conditions is tremendous. The animal is apparently able to conserve and store carbohydrate in spite of extended periods of fasting and integrate other energy-yielding

compounds from both exogenous and endogenous sources into metabolic pathways in such a manner that, even under the artificial stresses of fasting and phloridzin imposed in these experiments, the deviations from normal are small.

Application of the principles established in monogastric animals to polygastric (ruminant) animals would imply a rapid disappearance of liver glycogen (5) and, thereby, a deficiency of readily available glucogenic substances under the conditions imposed in these experiments. However, the animal was apparently able to maintain near-normal blood levels of T.R.S. in spite of a sizable glucose drain (55.81 g. in 8 hr.) superimposed upon 48 to 72 hr. of fasting. After another 24-hr. period of fasting, plus an additional glucose drain, the animal could resist insulin shock and exhibit less blood ketones than were present the previous day. Such observations are direct evidence that the animal can maintain near-normal functioning of its nervous tissue at a T.R.S. level which would produce insulin shock in monogastric animals (15) and still have a readily available source of glucogenic substances, because no severe drop in blood T.R.S. levels was observed following a sudden glucose drain (phloridzin). Although no direct evidence was available in these experiments, to pinpoint the source of the glucogenic substance necessary to prevent a drop in T.R.S. level, liver glycogen is the most likely source, due to the rapidity with which it can be mobilized. Since this postulation requires the assumption of considerable glycogen stores in the liver, even after 72 hr. of fasting, which is a marked deviation from results observed in monogastric animals, it would be interesting to investigate it further via liver biopsy and glycogen analysis during similar experiments.

It is unlikely that the adrenal medullary response activated via the hormone epinephrine was functioning during the course of the experiments. The mechanism is brought into play by a psychic stimulation (3) and causes the fight-or-run response. However, the major reserve of glucogenic substance which this hormone can tap is liver glycogen and should, thereby, be limited by the conditions cited above. Another factor which seems to argue against this mechanism being brought into play in the experiments reported here is the calmness with which the steer submitted to the treatments applied, the ease with which blood samples were drawn via the jugular catheter, and the lack of unusual deviations in the hourly blood samples within an experiment and between similar experiments.

Attempts to assay for urinary nitrogen increases as a measure of protein mobilization and degradation due to adrenocorticosteroids were vitiated by the large volume of urine excreted. The urinary output ranged up to 13.73 liters per 8-hr. period and so diluted the urinary nitrogen that direct measurements were impossible. This high rate of urine output did not cause serious dehydration of the animal, since it was nearly balanced by the infusion of isotonic mannitol solutions. In the experimental period in which 13.73 liters of urine was voided, 11.50 liters of mannitol was infused into the jugular vein. Only during those experiments run when the environmental temperatures were above 80° F. did the steer show signs of thirstiness, or accept water from a bucket.

The response of the steer to insulin is quite unusual, since it parallels the response in diabetic humans. This animal failed to show any of the symptoms of insulin shock and had lower levels of ketone bodies in his blood after insulin treatment than during administration of phloridzin alone, even though the T.R.S. concentration was lower. This observation implies that the body tissues were more capable of metabolizing ketones or that ketone formation was less at this low T.R.S. level than at a higher, more normal level. Since only extrahepatic tissue is considered capable of metabolizing ketones (6, 7), and the major pathway of oxidation is considered to be via the tricarboxylic acid cycle, a carbohydrate sparker in the form of oxalacetic acid must be present in order to oxidize C_2 pieces resulting from the cleavage of ketone bodies. The most logical explanation for this somewhat contradictory situation is that the insulin probably decreased the threshold level required for the passage of glucose across the cell membranes and thus built up the reserve of glucose or glucogenic substance in the cells. Due to this reserve, the animal was actually more able to metabolize ketone bodies as fast as they were available at a low blood level of T.R.S. than at a normal level. However, as this reserve was dissipated the ketones began to build up at a rate faster than that observed with phloridzin.

The low levels of ketones in the urine reflect the low levels in the blood and reaffirm the ability of the kidney to resorb utilizable substrate up to a given threshold. A sizable portion of the spillage observed from low blood ketones may have been due to the high filtration of water and mannitol from the blood and a mechanical interference with resorption due to the volume of liquid passing through the kidney tubules.

The sudden and sharp decline in blood T.R.S. values following the infusion of isotonic mannitol and butyrate, and the closeness with which it parallels the decline observed under the influence of both insulin and butyrate, suggests that the drop in blood T.R.S. level may be mediated through the release of endogenous insulin from the pancreas. The possibility, of course, is no more than suggested by the data obtained in these trials, but as soon as a good test for insulin levels in the blood is available, it would be an interesting phenomenon to investigate. The similarity in the blood ketone and butyrate concentrations is also evidence which would tend to point toward this possibility, because T.R.S. levels dropped, ketone levels failed to rise as rapidly, and blood butyrate did not build up as high, implying a more rapid transfer of glucose across cell membranes and an increased ability to metabolize butyrate.

A sharp deviation from the response reported by others (1, 2, 16) to oral or intraruminal drip of butyrate was observed when the butyrate was infused into the jugular vein. In the experiments reported here, the ketone levels in the blood did not exceed normal levels, except in the case of butyrate superimposed on phloridzin. This contrasts sharply with levels of 35 to 70 mg/100 ml. which are reported following the introduction of proportionally smaller amounts of butyrate into the rumens of sheep and goats. This could be a species difference, but this is not probable. The high levels of ketones found in ketotic cows would indicate that the bovine can accumulate ketone bodies under certain conditions. The primary difference in the two methods of butyrate

administration seems to be concerned with the rumen. Pennington (12, 13) reports that, in studies in which ruminal epithelium is incubated with butyrate as the sole substrate, large quantities of ketones are formed. Numerous workers (9, 17) have projected this observation and state that butyrate is converted to β-hydroxybutyrate during its passage through the rumen wall. This is hardly enough evidence to establish such a mechanism as operative in an intact animal whose ruminal epithelium is exposed to all of the substrates available in the circulating blood. Rumen perfusion data (19) show that when the concentration of butyrate in rumen fluid is increased to high levels by the injection of butyric acid, low levels of ketones appear in the perfusate, but these are quite insignificant when compared to the amount of free butyrate which is absorbed into the blood. In the course of the perfusion the ruminal tissue uses considerable quantities of glucose from the perfusate, casting some doubt on the reliability of single substrate incubations when projected into intact systems.

The buildup of butyrate in the jugular blood is also a sharp deviation from results observed when butyrate is dripped into the rumen. If butyrate is absorbed unchanged in the intact animal, as it is in the perfused rumen, it would be expected that it would be transported to a large degree as the sodium salt due to the pH of blood and sodium being the major extracellular base. This would imply little or no difference in the form in which the butyrate would be available at the tissue level and gives little cause for this difference. The most obvious differences are amount of butyrate supplied and its route of entry into the system.

For the amount to be important, it would have to be postulated that the system was unable to metabolize butyrate at the rate at which it was infused during the early part of the experiment but, during the period, was able to increase its metabolic capacity for butyrate and, thus, by the end of the period, reduce the blood butyrate level from its peak to a level which it could maintain when butyrate metabolism was maximum. It also appears that such a maximum rate of butyrate metabolism can be established faster even at a similar level of blood T.R.S. if the glucose drain is nonexistent or reduced somewhat as in the case of Experiments 5 and 7. Another interesting difference between the methods is the route of travel of the butyrate after its introduction into the body. In the case of the ruminal drip, all newly introduced butyrate would be transported directly to the liver via the portal vein, whereas with jugular infusion the butyrate would be distributed randomly throughout the circulatory system and would not be presented directly to the liver. Under normal conditions butyrate does not appear in large amounts in the blood that is coming from the liver and the predominant V.F.A. present in peripheral blood is acetate. If no increase in jugular blood butyrate follows its intraruminal drip, then this system apparently has a large capacity and removes butyrate completely. However, this difference in route of passage does not explain the early peak concentration of butyrate followed by a reduction to a lower level at which it stabilized. This would imply an increase in the rate of butyrate metabolism.

The fact that this steer was able to maintain normal function throughout an 8-hr. infusion period in which a total of 200 g. of butyrate was infused is an interesting observation concerning the bovine's ability to utilize butyrate. Since less than 15 g. of the infused butyrate could be accounted for as blood butyrate and ketones or urinary ketones, it must be assumed that the remainder of the dose infused (185 g.) was metabolized during the period. No direct evidence concerning the form in which it became available to the tissues was obtained in this experiment. However, if it became available as activated 2-carbon fragments (acetyl coenzyme A), it was rapidly utilized in this form and no significant amount of it was inactivated and released as free acetate into the blood because blood acetate remained low throughout the experiments. However, this does not rule out the release of inactivated 2-carbon fragments by the liver, transport by the blood to extrahepatic tissue, and reactivation and utilization there at a rate fast enough to prevent an increase to normal levels in the blood.

Similar reasoning would imply that if butyrate is oxidized to aceto-acetic acid (7) and metabolized as such, its removal from the blood is rapid enough that there is no large increase in blood ketones.

The most pertinent observation from these experiments, when considered as a whole, is that a simple glucose stress of the magnitude induced in these experiments is not sufficient to cause the clinical symptoms of bovine ketosis, even when superimposed on up to 96 hr. of fasting and high infusion rates of a ketogenic substance (butyrate). Therefore, experimental production of clinical symptoms of bovine ketosis is dependent upon either more severe glucose stress or some other key trigger mechanism.

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EFFECT OF DURATION OF DEFICIENT CAROTENE INTAKE UPON SUBSEQUENT UTILIZATION OF CAROTENE FROM ALFALFA BY HOLSTEIN CALVES [1]

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SUMMARY

Thirty-two Holstein male calves, approximately 63 days of age, were fed a vitamin A depletion ration at an intake to provide an anticipated seven-day increase in live weight of 10 lb. until partially depleted of their vitamin A stores. When the average plasma vitamin A concentration was 8.0 ± 1.8 γ per 100 ml., each calf was then fed in addition to the vitamin A depletion ration, one of two initial period carotene intakes per pound of live weight per day, 12- or 48-γ, for one of two durations, 4- or 16-wk. Each calf was then changed to one of two final period carotene intakes, 60- or 240-γ. Durations for the final periods were either 24 wk. for the calves fed the initial period carotene intake for 4 wk., or 12 wk. for the calves fed the initial period carotene intake for 16 wk. During the terminal seven-day period of the initial 16-wk. carotene intake period, only those calves fed the 12-γ carotene intake had cerebrospinal fluid pressures above 120 mm. of saline. During the terminal seven-day period of the 12-wk. final carotene intake period, those calves fed the 12-γ initial period carotene intake and the 60-γ final period carotene intake had pressures greater than 120 mm. but of smaller magnitude than observed during the initial carotene intake period. Based upon blood plasma and liver vitamin A concentrations, determined on samples obtained terminally during the final carotene intake period, calves fed the 12-γ initial carotene period intake for 16 wk. utilized the 60- and 240-γ carotene intakes 0.6 to 0.8 as effectively as calves fed 48-γ carotene intake initially. While the differences between the 12- and 48-γ carotene for the 4-wk. duration were slight, across both the 4- and 16-wk. durations, calves fed the 12-γ intake utilized carotene between 0.7 and 0.9 as efficiently as calves fed the 48-γ intake.

Cattle are subjected, at times, to periods of suboptimum carotene intake which, if continued, result in vitamin A deficiency. There is some evidence to indicate that cattle fed deficient carotene intakes for extended periods of time, or cattle fed carotene-free rations and exhibiting extremely low plasma and liver vitamin A concentrations, do not utilize carotene as efficiently as cattle previously fed rations adequate with respect to carotene intake. For example, Byers and coworkers of the Oregon Station (1) fed adequate and suboptimum

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carotene intakes to Jerseys and Holsteins prior to and after calving, and observed low liver concentrations of carotene and vitamin A, determined on biopsy samples, in the suboptimum carotene intake animals. Subsequent feeding of higher levels of carotene, or repeated injections of vitamin A to cows in the suboptimum carotene intake group, resulted in inappreciable increases in liver vitamin A stores. The lack of response was interpreted as a decreased ability of the animal to utilize either carotene or preformed vitamin A when previously fed suboptimum carotene intakes. No increase was found by Erwin *et al.* (5) in the vitamin A concentrations of biopsy samples from the livers of steers depleted to an average plasma vitamin A level of 2 γ per 100 ml., and, subsequently given by stomach tube an aqueous dispersion of carotene at the rate of 15 mg. of carotene per 100 lb. live weight on alternate days for a ten-day period. Upon similar administration of carotene to steers having an average initial plasma vitamin A value of 65 γ per 100 ml., a marked increase in liver vitamin A stores was observed. In contrast, plasma vitamin A values increased tenfold in the depleted steers and changed inappreciably in the normal steers. In a similar short-time study, Diven and Erwin (3), using wethers having an average initial plasma vitamin A value of approximately 25 γ per 100 ml. and given intraruminally a single dose of 6 mg. of carotene in an aqueous dispersion, had considerably greater increases in liver concentrations of vitamin A than deficient sheep with average initial values of approximately 10 γ per 100 ml. Approximately a 10 γ per 100 ml. increase in the concentration of vitamin A in the plasma was found for both the normal and the deficient sheep.

Since in the aforementioned studies, cattle were either fed deficient carotene intakes for extended periods of time (1) or were fed essentially a carotene-free ration until practically devoid of vitamin A stores (5), the present study was undertaken to extend these findings by including as a variable, duration of deficient carotene intake, and testing its effect upon subsequent carotene utilization.

EXPERIMENTAL PROCEDURE

Animals and feeding. Thirty-two male one-day-old Holstein calves from Connecticut state institution herds were obtained during the period September-November, 1958, and placed in individual sawdust-bedded tie-stalls in a portion of the research barn in which neither light nor temperature was controlled. Upon arrival at the barn, each calf received one 500-mg. oblet of chlortetracycline (Lederle's Aureomycin) as a prophylaxis for scours and one gelatin capsule containing 100,000 U.S.P. units of vitamin A (Nopcay "250", Type M, guaranteed to contain 250,000 U.S.P. units of vitamin A per gram) to insure adequate vitamin A storage. Thereafter, each was raised to approximately 63 days of age on a limited whole milk, limited vitamin A depletion grain mixture, and ad libitum chopped alfalfa hay as described previously (4), except that alfalfa was added during this period at the rate of 5 to 100 lb. of the depletion grain mixture to supply adequate carotene intake.

On approximately the 64th day of age, each calf received a vitamin A depletion ration, consisting of one-third beet pulp and two-thirds vitamin A

depletion grain mixture (but no alfalfa was added as in the previous period), at a rate of intake to provide an anticipated 10-lb. increase in live weight per seven-day period. When the blood plasma concentration of vitamin A, based on blood samples obtained at successive seven-day periods, had decreased to equal to or less than 12 γ per 100 ml., each calf was then fed, after completion of the next seven-day period, one of two initial carotene intakes from artificially dehydrated alfalfa leaf meal, 12 or 48 γ per pound of live weight per day, for one of two periods, 4- or 16-wk. Upon completion of this initial carotene supplementation period, each calf was then fed either the 60- or 240-γ carotene intake, with the length of this final carotene intake period being 24 wk. for those fed the initial carotene intake for 4 wk., and 12 wk. for those fed the initial carotene intake for 16 wk. The levels of initial carotene intake and duration of feeding were selected after consideration had been given to the data from this Station as well as others (8) on the requirement for carotene necessary to prevent vitamin A deficiency change(s), as well as the length of time required for the appearance of this change(s). Also, since initial vitamin A status (as indicated by plasma and liver vitamin A concentrations) has recently been demonstrated to affect the response to subsequent carotene intake in both calves and steers (7, 9) which were apparently not vitamin A deficient, the initial carotene intakes were chosen to be narrowly distributed but yet resulting in anticipated vitamin A deficiency change(s) at the lowest intake when fed for the 16-wk. duration. The levels of final carotene intake were based upon additional data (4, 11), such that linear responses of plasma and log liver vitamin A concentrations on log carotene intake would be expected and, thus, the data would be suitable for quantitative estimation of the effects of periods of vitamin A deficiency upon subsequent carotene utilization by employment of parallel bioassay procedures.

Assignment of calves to their respective treatments was according to random allotment determined prior to their arrival at the research barn. However, this assignment was in blocks of eight calves each, with the first eight calves to arrive making up the first block and so forth. Average age at the start of the initial carotene supplementation period was 81 days with a standard deviation of five. One or three days following the completion of the initial and final carotene supplementation periods, a total of 28 wk., each calf was slaughtered.

Calculation of the amount of alfalfa fed to supply the desired carotene intake, incorporation of the alfalfa into the vitamin A depletion ration, and treatment for scours were similar to previous studies from this Station (8). However, to prevent deviations from each calf's fixed carotene intake such as experienced recently (4), the designated amount of alfalfa, made up to 1 or 2 lb. with vitamin A depletion ration, was fed separately first in the morning. After the calf had entirely consumed this, the remainder of its vitamin A depletion ration allowance was then fed. Average minimum and maximum daily temperatures observed during both periods of carotene supplementation were, with their standard errors, respectively, 53.5 $\pm$ 0.4° F. and 64.9 $\pm$ 0.5° F. Average light intensity, natural and artificial, measured in most cases

at noon at successive 4-wk. periods at a height of 48 in. in the center of each calf stall, was 9.6 ± 1.0 foot-candles.

Observations and analyses. These were identical to those reported for another study (4) from this Station, with the exception of the measurement of cerebrospinal fluid pressure during the terminal seven days of the initial and final carotene intake periods. The method of pressure measurement has been described in a subsequent publication (2) and is essentially the manometric procedure of Sykes and Moore (10). In Table 1, average chemical composition of the feeds fed are presented, including carotene content.

TABLE 1
Average chemical composition of feeds

Feed	Dry matter	Crude protein	Ether extract	Crude fiber	NFE	Ash	Carotene
	(%)	(% dry matter)					(mg/lb)
Alfalfa hay, chopped	92.1 ± 0.6[a]	19.6 ± 0.7	2.2 ± 0.4	32.3 ± 0.9	38.3 ± 1.0	7.6 ± 0.4	4.5 ± 1.0
Vitamin A depletion grain mixture	91.0 ± 0.6	19.2 ± 0.7	4.0 ± 0.3	7.6 ± 0.2	62.3 ± 0.3	6.9 ± 0.3	0.6 ± 0.3
Vitamin A depletion ration	90.2 ± 0.5	16.2 ± 0.4	2.6 ± 0.2	12.1 ± 0.3	62.5 ± 0.4	6.6 ± 0.2	0.1 ± 0.0
Artificially dehydrated alfalfa leaf meal	90.1 ± 1.0	23.7 ± 0.4	4.5 ± 0.3	19.9 ± 0.6	39.7 ± 1.0	12.2 ± 0.2	93.1 ± 1.3

[a] Standard error of the mean.

The data were subjected to analysis of variance for the purpose of isolating variability among blocks of calves, among treatments and remainder (error). Prior to the beginning of the experiment, the among treatments variability was further divided into two sets of contrasts. The first included that variation due to initial period carotene intakes, to durations of initial period carotene intake, to final period carotene intakes, and to their interactions. The second, intended only for plasma and liver vitamin A concentrations, was for the purpose of testing the validity of the use of parallel line bioassoy procedures in estimating the effects of initial carotene intake and its duration upon subsequent carotene utilization (i.e., the utilization of the 60- and 240-γ intakes). It included variation due to: a) treatments other than final period carotene intakes, b) the over-all linear response on final period carotene intakes (60- and 240-γ), c) differences in the linear responses among those calves fed either the 12- or 48-γ initial period carotene intakes each for the 4- or 16-wk. durations, d) differences in the linear responses between the two initial period carotene intakes, 12 and 48 γ, and e) differences in the linear responses between the two initial period carotene intake durations, 4 and 16 wk.

RESULTS AND DISCUSSION

Feed consumption, growth, health, and cerebrospinal fluid pressure. Consumption of the vitamin A depletion ration allowance during the initial and

TABLE 2

Effect of periods of deficient carotene intake on feed consumption, live weight gain, and cerebrospinal fluid pressure of Holstein calves [a]

Treatments				Days consuming ration allowance	Live weight		Cerebrospinal fluid pressure			
Initial period		Final period			Prior to carotene supplementation	Gain [d]	Initial period terminal value		Final period terminal value	
Intake	Duration	Intake	Duration				Actual	Log	Actual	Log
(γ) [b]	(wk.)	(γ) [b]	(wk.)	(%)	(lb.)		(mm. saline)			
12	4	60	24	98.2(84.0) [c]	186	412(421) [e]	84	1.90	108	1.97
12	4	240	24	98.6(85.3)	184	423(434)	62	1.77	67	1.83
12	16	60	12	99.6(88.2)	204	438(436)	186	2.20	161	2.17
12	16	240	12	99.1(86.4)	204	423(420)	141	2.13	64	1.80
48	4	60	24	100.0(90.0)	206	457(453)	72	1.84	82	1.91
48	4	240	24	99.6(88.2)	202	429(428)	84	1.90	78	1.89
48	16	60	12	100.0(90.0)	212	450(441)	82	1.91	67	1.82
48	16	240	12	99.5(88.0)	202	415(414)	61	1.69	88	1.93
Standard deviation per calf				— (4.0)	28	30(25)	....	0.22	....	0.15

[a] Values represent four calves per treatment group.
[b] γ carotene per pound of live weight per day.
[c] Arc sin $\sqrt{Y}$, in which Y = per cent of total days, 196.
[d] Final period terminal value minus value obtained the day prior to carotene supplementation.
[e] Values adjusted by covariance for live weight the day prior to carotene supplementation.

final carotene intake periods (Table 2) was maintained at a relatively high level. However, calves fed the 12-γ initial period carotene intake consumed slightly less of their ration allowance (particularly, during the final carotene intake period) than calves fed the 48-γ initial period carotene intake. This difference was found to be statistically significant ($P < 0.05$) upon analysis of variance of the transformed percentages.

Growth of the calves, as indicated by live weight increases (Table 2), was not appreciably affected by treatment.

Health problems were minimal during the initial and final carotene intake periods. During these periods, only two calves, both fed the 12-γ initial period carotene intake for 16 wk., followed by the 60-γ final period carotene intake, exhibited scours for two days and one day, respectively. No calves required treatment for scours during either carotene intake period.

Cerebrospinal fluid pressures (Table 2) taken during the terminal seven days of the initial carotene intake period, were greater for calves fed the 12-γ intake (average across all groups receiving this intake) than those for calves fed the 48-γ intake. Since calves fed the 12-γ initial period carotene intake for only a 4-wk. duration had pressures differing only slightly from the groups fed the 48-γ carotene intake for durations of either 4 or 16 wk., the difference in pressures between initial carotene intakes was largely attributable to the group receiving the 12-γ intake for the 16-wk. duration ($P < 0.05$ for the interaction of initial period carotene intakes and durations of initial period carotene intake). Cerebrospinal fluid pressures, observed during the terminal seven days of the final carotene intake period, were greater in those calves fed the 12-γ initial carotene intake for the 16-wk. duration and the 60-γ final carotene intake than in the other treatment groups ($P < 0.10$ for final period carotene intakes and $P < 0.01$ for the interaction of initial period carotene intakes and final period carotene intakes).

Using cerebrospinal fluid pressures of greater than 120 mm. of saline as indicative of vitamin A deficiency (6), it was found that the feeding of as little as 12-γ carotene per pound of live weight per day for a period of 4 wk. did not result in deficiency. However, extending this period to 16 wk. resulted in deficiency of such severity that the subsequent feeding of 60-γ carotene intake for a 12-wk. period, approximately twice the intake necessary to prevent elevated cerebrospinal fluid pressure in Holstein calves (6), did not result in the pressures returning to the normal level of 120 mm. or less.

Carotenoids and vitamin A. In Table 3 are given plasma carotenoids and vitamin A concentrations on the day prior to carotene supplementation, terminal concentrations for the initial carotene intake period, and average and terminal values for the final carotene intake period. Concentrations of the same constituents for liver obtained at the completion of the final carotene intake period are given in Table 4.

Initial period terminal plasma carotenoid values were greater in those calves fed the 48-γ carotene intake (average across all groups receiving this intake) than in those fed the 12-γ intake. Also, the duration of the initial period

TABLE 3

Effect of periods of deficient carotene intake on plasma carotenoids and vitamin A concentrations of Holstein calves [a]

Treatments				Plasma carotenoids				Plasma vitamin A			
Initial period		Final period				Final period				Final period	
Intake	Duration	Intake	Duration	Prior to carotene supplementation	Initial period terminal value	Av.	Terminal value	Prior to carotene supplementation	Initial period terminal value	Av.	Terminal value
(γ)[b]	(wk.)	(γ)[b]	(wk.)	(γ/100 ml.)							
12	4	60	24	9	8	51	68(1.82)[c]	7.3	5.2	17.0	18.4
12	4	240	24	19	9	122	151(2.17)	8.5	6.0	23.2	22.9
12	16	60	12	10	14	59	73(1.86)	5.6	5.0	15.1	15.6
12	16	240	12	8	10	134	149(2.17)	9.6	6.5	23.8	23.8
48	4	60	24	9	22	44	50(1.69)	7.8	13.1	17.3	18.0
48	4	240	24	15	26	150	200(2.29)	8.2	12.0	25.1	25.3
48	16	60	12	10	47	61	71(1.85)	7.8	17.7	19.9	20.6
48	16	240	12	16	38	143	155(2.18)	9.0	15.8	24.9	23.7
Standard deviation per calf				....	....	....	....(0.09)	1.8	2.3	3.1	3.5

[a] Values represent four calves per treatment group.
[b] γ carotene per pound live weight per day.
[c] Log γ carotenoids per 100 ml.

TABLE 4

Effect of periods of deficient carotene intake on liver carotenoids and vitamin A concentrations of Holstein calves [a]

Treatments									
Initial period		Final period			Liver carotenoids		Liver vitamin A		Liver vitamin A per lb. of live weight
Intake	Duration	Intake	Duration	Liver weight	Actual	Log	Actual	Log	
(γ)[b]	(wk.)	(γ)[b]	(wk.)	(g.)	(γ/100 g.)				(log γ/lb)
12	4	60	24	3,671	89	1.94	246	2.32	1.11
12	4	240	24	3,724	192	2.28	2,528	3.40	2.20
12	16	60	12	3,760	88	1.94	204	2.29	1.06
12	16	240	12	4,014	238	2.37	1,626	3.21	2.01
48	4	60	24	4,027	89	1.94	244	2.31	1.08
48	4	240	24	3,967	242	2.37	2,251	3.39	2.14
48	16	60	12	4,093	101	2.00	375	2.53	1.32
48	16	240	12	3,883	205	2.30	2,000	3.29	2.09
Standard deviation per calf				330	....	0.09	....	0.21	0.20

[a] Values represent four calves per treatment group at the completion of each calf's 28-wk. supplementation period.
[b] γ carotene per pound of live weight per day.

affected these values, since the values for those calves on the 16-wk. duration were slightly greater than the values for those calves on the 4-wk. duration. This effect of duration of initial period carotene intake was also evident in the average plasma carotenoid values observed during the final carotene intake period. As was expected, there was a relatively marked effect of final carotene intake upon plasma carotenoids observed during the final carotene intake period.

The average terminal plasma vitamin A concentration for the initial carotene intake period was approximately twice as high in calves fed the 48-γ intake as compared to that in calves fed the 12-γ intake. The 16-wk. duration of this initial carotene intake period resulted in slightly greater values in calves fed the 48-γ intake than those in calves fed the same intake but for only 4 wk. duration. Besides the marked effect of final carotene intakes on average and terminal values of this criterion during the final carotene intake period, those calves receiving the 12-γ initial carotene intake tended to maintain lower values than those fed 48 γ. The latter effect was pronounced in the group fed initially the 12-γ intake for the 16-wk. duration and the 60-γ final period carotene intake.

The responses to final carotene intake of both carotenoids and vitamin A concentrations in the livers of all calves were marked, but the carotenoid values were inappreciably affected by other treatments imposed during the initial carotene intake period. Vitamin A values tended to be lower in calves previously fed the 12-γ carotene intake and, as for plasma vitamin A, this effect was most marked in calves previously fed this intake for the 16-wk. duration.

To quantify the effects of periods of deficient carotene intake upon subsequent carotene utilization, linear regression coefficients of plasma and liver vitamin A concentrations on log γ carotene intake, 60 and 240 γ, during the

final carotene intake period were derived and are presented in Table 5. These coefficients agreed reasonably well with those previously reported (4, 11). While there was a tendency for the coefficients of the group of calves fed the initial period carotene intake of 48 γ for the 16-wk. duration to be of smaller magnitude than the other treatment groupings, it was found that the coefficients for either of these criteria did not differ significantly (at $P \leq 0.05$) from the other coefficients. Thus, under the assumption that the responses to log carotene intake were parallel, the relative effects of periods of deficient carotene intake were determined by calculating the horizontal distance between any two parallel lines of response of these criteria. The antilogarithm of this distance represents the ratio of carotene intakes which result in equivalent response.

In Table 6 are presented the relative effects of periods of deficient carotene intake upon utilization of carotene, 60- and 240-γ intakes. The feeding of 12 γ of carotene for a period of 4-wk. duration had little effect on subsequent utilization of carotene, as evidenced by ratios slightly less than unity for plasma vitamin A concentrations and by ratios slightly greater than unity for liver vitamin A concentrations. In contrast, the feeding of the same initial carotene intake, but for a 16-wk. duration, resulted in decreased utilization (i.e., ratios considerably less than unity). The effects of initial period carotene intake, 12 versus 48 γ, averaged across both durations (third row of Table 6)

TABLE 5

Effect of periods of deficient intake upon subsequent response, linear regression coefficients of response criteria on log carotene intake, to 60 and 240 γ carotene intakes [a]

Initial period treatments		Response criteria			Liver vitamin A per lb. of live weight
		Plasma vitamin A		Liver vitamin A	
Intake	Duration	Final period average	Final period terminal		
(γ)[b]	*(wk.)*	*(γ/100 ml.)*		*(log γ/100 g.)*	*(log γ/lb)*
12	4	10.2 ± 3.6[c]	7.5 ± 4.1	1.79 ± 0.25	1.81 ± 0.25
12	16	14.4 ± 3.6	13.4 ± 4.1	1.53 ± 0.25	1.59 ± 0.25
48	4	13.0 ± 3.6	12.1 ± 4.1	1.80 ± 0.25	1.75 ± 0.25
48	16	8.2 ± 3.6	5.2 ± 4.1	1.26 ± 0.25	1.29 ± 0.25
12	4+16	12.3 ± 2.5	10.5 ± 2.9	1.66 ± 0.18	1.70 ± 0.17
48	4+16	10.6 ± 2.5	8.6 ± 2.9	1.53 ± 0.18	1.52 ± 0.17
12+48	4	11.6 ± 2.5	9.8 ± 2.9	1.79 ± 0.18	1.78 ± 0.17
12+48	16	11.3 ± 2.5	9.3 ± 2.9	1.39 ± 0.18	1.43 ± 0.17
All data combined		11.4 ± 1.8	9.6 ± 2.0	1.59 ± 0.12	1.61 ± 0.12

[a] All linear regression coefficients positive. First four rows of coefficients based on eight calves each, second four rows based on 16 calves each, and final row on 32 calves.
[b] γ per pound live weight per day.
[c] Standard error of slope.

TABLE 6

Relative effects (ratios) of periods of deficient carotene intake upon subsequent utilization of carotene by Holstein calves

	Criteria			
	Plasma vitamin A		Liver vitamin A	
	Final period average	Final period terminal value	Final period terminal value	Final period terminal value per lb. of live weight
	(γ/100 ml.)		*(log γ/100 g.)*	*(log γ/lb)*
Ratio of 12-γ intake to 48-γ for 4-wk. duration of initial period	0.80 0.39–1.56[a]	0.77 0.27–1.95	1.01 0.74–1.40	1.07 0.79–1.46
Ratio of 12-γ intake to 48-γ for 16-wk. duration of initial period	0.55 0.25–1.06	0.56 0.17–1.36	0.79 0.57–1.09	0.79 0.57–1.07
Ratio of 12-γ intake to 48-γ for both 4- and 16-wk. initial periods	0.66 0.38–1.05	0.66 0.29–1.23	0.90 0.71–1.12	0.92 0.74–1.14
Ratio of 16-wk. duration of initial period to 4-wk. duration	1.05 0.65–1.72	0.94 0.47–1.86	0.96 0.77–1.21	0.99 0.79–1.23

[a] Ninety-five per cent confidence limits.

resulted in a relatively smaller effect. The effects of duration of initial period carotene intake, 16 versus 4 wk., averaged across both initial carotene intakes, were quite small and of questionable importance.

The data with respect to the effects of deficient carotene intake upon subsequent carotene utilization, as evidenced by plasma and liver vitamin A concentration of calves fed alfalfa as a source of carotene, are interpreted as essentially confirming the experiments reviewed initially in this paper. However, upon comparing the data on the effects of deficient carotene intake of 4-wk. duration to those of 16-wk. duration, it is evident that the duration of deficient carotene intake is a parameter of importance in the evaluation of data from such studies. In the application of the statistics contained herein, it is necessary to recognize, as discussed previously (9), that they are point-in-time estimates. Since the criteria used, plasma and liver vitamin A concentrations, are subject to previous plasma and liver vitamin A concentrations, current carotene intake, and other environmental influences too numerous to document here, as well as previously cited (4) genetic effects, the only valid generalization is that the duration of deficient carotene intake needs to be considered as a variable in evaluation of the vitamin A status of calves subsequently fed carotene.

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A COMPARISON OF FEEDING GROUND CORNCOBS AND A LIQUID UREA-MOLASSES-MINERAL MIX WITH AND WITHOUT ETHYL ALCOHOL TO YEARLING DAIRY HEIFERS[1, 2]

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SUMMARY

Twenty dairy heifers, ten Holsteins and ten Jerseys, were divided into two equal groups and were individually fed ad libitum but separately a ration of ground corncobs and a liquid urea-molasses mix with and without ethyl alcohol during a 140-day trial. Group A received the mix without ethyl alcohol, and Group M received the mix with ethyl alcohol. One Jersey heifer was continued on each ration for an additional 438 days. During the 140-day trial the average daily gains were 0.80 and 0.73 lb. for Groups A and M, respectively. This difference was not statistically significant. A large variation in growth rates of the heifers existed in both groups. Groups A and M consumed 15.0 and 14.3 lb. of corncobs and 4.8 and 5.5 lb. of liquid mix per animal per day, respectively. Water consumption was similar for both groups, whether offered water continuously or once a day. Blood urea and plasma NPN values were significantly higher for Group M. Rumen volatile fatty acid values were similar and normal for both groups, indicating normal rumen fermentation. Animals were able to grow, mature, and produce milk on rations in which essentially all of the digestible protein equivalent was supplied by urea without any apparent toxic effect.

A liquid mix composed primarily of urea, phosphoric acid, water, denatured ethyl alcohol, and molasses has recently become available for commercial use. It has been recommended for feeding to ruminant animals as a satisfactory supplement and is designed to be fed in the liquid state. This mix could conceivably supply a large part of the protein equivalent requirement in many rations.

Since this study was conducted, Morris and Horton (5) reported that three groups of dairy heifers fed an alcohol-molasses-urea preparation along with 1 lb. of ground corn and cottonseed hulls, native grass hay and 2 lb. of ground corn, and cottonseed hulls and 3 lb. of cottonseed oil meal–corn concentrate mix led to average daily gains of 0.88, 1.26, and 0.96 lb., respectively. Later reports indicated that ethyl alcohol added to either a urea–blackstrap molasses mix or an ammoniated–blackstrap molasses mix did not provide any special stimulus for gain by steers (7), and nitrogen retention was lower on a grass hay molasses-urea mineral ration than on other rations where some or all soybean oil meal was fed instead of urea (4). Ethanol was no more effective than purified corn starch or corn meal in increasing urea utilization.

This study, briefly reported previously (1), was designed to determine: (a) whether urea as essentially the sole source of digestible protein could support

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normal growth in dairy heifers; (b) the effect of high levels of urea intake upon the blood urea and nonprotein nitrogen, and plasma protein levels of dairy heifers; (c) if feeding urea at these levels would cause urea toxicity; (d) whether ethyl alcohol would stimulate increased gains; (e) if ethyl alcohol would cause an altered rumen bacterial metabolism as indicated by rumen volatile fatty acid concentrations.

EXPERIMENTAL PROCEDURE

During the fall of 1956, ten Holstein and ten Jersey heifers were divided into two comparable groups and fed two experimental rations, hereafter referred to as Rations A and M.

This experiment consisted of five 28-day periods following a 28-day preliminary experimental period.

Experimental rations and management. Corncobs ground to pass through a 3/8-in. screen and fed ad libitum served as the only roughage and the only dry feed in both rations. Group A received Liquid Mix A and Group M received Liquid Mix M ad libitum (Table 1). All animals were fed individually

TABLE 1
Composition of liquid mixes

Liquid mix	A	M
	(%)	
Ingredients		
Urea	10.0	10.0
Minerals	3.5	3.5
Molasses, blackstrap	72.7	66.7
Water	13.9	13.9
Denatured 95% ethyl alcohol	0.0	6.0

and were completely switched to the experimental rations the first day of the experiment.

The ground corncobs were weighed and fed daily at a rate to endeavor to keep them before the heifers at all times.

The liquid mixes were weighed each day into a 10-qt. galvanized container for each heifer. The container was then placed in one corner of the manger and held in place by a metal strap to prevent spillage.

Ground corncobs were employed for bedding to prevent the consumption of bedding. All animals were released once daily, except when water consumption was studied, into a dry lot where they had access to water, steamed bonemeal, and NaCl.

Water consumption. Three animals in each group, two Jerseys and one Holstein, were offered water continuously for 22 days. The water consumption of the seven remaining animals in each group, offered water once a day from a metal tank, was measured daily.

Blood analyses. Blood samples were taken from five Jerseys and five Holsteins twice each period. Whole blood urea was determined by the Van Slyke and Cullen method modified to use boric acid (2). Total plasma nitrogen and

nonprotein nitrogen were determined by the micro-Kjeldahl distillation method (2).

Rumen volatile fatty acids. Three rumen samples were taken at weekly intervals from each animal during the fourth and fifth periods and volatile fatty acids were quantitatively determined for each sample. The method used was the direct chromatographic determination of C_1 to C_6 fatty acids in rumen fluid as outlined by Keeney (3).

Additional feeding. Two mature, fistulated steers, one Jersey and one Holstein, were placed on experiment for 252 days. The Jersey received Ration A and the Holstein received Ration M. One Jersey heifer from each group was maintained on the same rations for an additional 438 days.

RESULTS AND DISCUSSION

Body weight changes. The average daily gains for the heifers, during the 140-day experiment, were 0.80 and 0.73 lb., respectively, (Table 2) for Groups A and M. Analysis of variance (8) indicated that this difference was not significant.

The urea, as a protein substitute, furnished essentially all the nitrogen of the digestible protein equivalent in the rations, since cane molasses and corncobs reportedly contain no digestible protein (6). This trial did show that at least some of the animals in both groups could make normal gains on a ration in which all the digestible protein equivalent was supplied in the form of urea. The average daily gains made by the individual animals were quite variable. Average daily gain for heifers of Group A ranged from 0.20 to 1.18 lb. and from 0.18 to 1.24 lb. for those of Group M (Table 2). The variation in gain can not be explained on the basis of ratio of consumption of liquid mix to corncobs. Neither can the variation be explained on the basis of total feed consumption in all cases.

The average daily gain by breed was 0.99 and 0.60 lb. per day for the Holstein and Jerseys, respectively, for Group A, and 0.77 and 0.70 lb. per day for the Holsteins and Jersey of Group M.

Feed consumption. The consumption of corncobs and liquid mix was relatively consistent throughout the entire trial. The average daily consumption of cobs was 15.0 lb. for Group A and 14.3 lb. for Group M and the average daily consumption of mix was 4.8 lb. and 5.5 lb. for Groups A and M, respectively. These differences were not statistically significant. The Holsteins consumed a significantly ($P < 0.01$) greater amount of cobs and liquid mix than did the Jerseys, as would be expected because of their larger size. Group A required 24.8 lb. of total ration to produce a pound of gain as compared to 27.1 lb. for Group M. The heifers consumed a total of 101.2 lb. of NaCl and 75.6 lb. of steamed bonemeal.

The normal rate of gain for heifers of this size and age according to Morrison's (6) standard would be approximately 1.2 lb. per day for the Holsteins, and 0.8 lb. per day for the Jerseys. The approximate Morrison TDN requirement for these heifers to make this rate of gain would be 11.2 lb. for the Hol-

TABLE 2
Animal performance for 140 days

Animal No.	Body weight gain	Ground corncob consumption	Mix consumption
	(lb/day)		
	Group A		
182H	1.13	18.25	4.35
193H	0.61	17.53	6.84
194H	1.03	17.47	5.64
208H	1.18	17.88	5.87
216H	1.01	16.45	6.18
185J	0.20	12.51	3.91
191J	0.33	12.05	3.47
209J	1.12	13.07	4.02
212J	0.59	13.30	4.05
222J	0.78	11.60	3.51
Daily average per animal	0.80	15.01	4.78
	Group M		
178H	1.01	16.80	4.97
184H	0.76	17.41	6.54
199H	0.25	15.98	7.82
213H	0.58	18.33	7.60
220H	1.24	16.18	7.00
189J	0.86	14.43	5.07
200J	0.69	11.27	3.78
196J	0.18	10.68	4.58
219J	0.67	10.81	3.62
227J	1.09	11.50	4.23
Daily average per animal	0.73	14.33	5.52

steins, and 9.4 lb. for the Jerseys. The TDN consumption was 7.20 lb. per day for the Jerseys in Group A. The Holsteins in Group A consumed 10.27 lb. of TDN per day. The Jerseys and Holsteins both consumed less estimated TDN than would be required to make the rate of gain indicated above. For Group M, the Jerseys consumed 7.26 lb. of TDN per day and the Holsteins consumed 10.74 lb. of TDN per day. It is not surprising that somewhat less than normal growth of the heifers was obtained, since both groups consumed less energy than necessary for normal growth.

Water consumption. Limited data from the 22-day observation indicated that water consumption was similar (47 *vs.* 46 lb/animal/day) for the two different rations. Those watered once a day consumed per 1,000 lb. of body weight 55.6 lb. of water per day and those watered continuously consumed 61.2 lb. per day. This difference was statistically significant ($P < 0.01$). The difference remained significant when water consumption was adjusted to the 0.75 power of body weight. These very limited data indicated that continuous watering led to a greater water consumption than watering once daily.

Blood analyses. The blood urea, plasma nitrogen, and nonprotein nitrogen values were within the normal range. The average urea content of Group A was 14.08 mg.%, and 16.89 mg.% for Group M, the difference being statistically significant ($P < 0.01$). The average plasma protein levels were 5.74 and 5.80 g. per 100 ml. of plasma for Groups A and M. This difference was not significant. The Holsteins had significantly ($P < 0.05$) higher plasma protein levels than

did the Jerseys. Nonprotein nitrogen levels averaged 29 and 32 mg. per 100 ml. of plasma for Groups A and M, respectively. This difference was significant ($P < 0.05$).

Rumen fatty acids. The results of the rumen fatty acid analyses are given in Table 3. There was no significant difference between groups in any of the fatty acid fractions.

Incidence of toxicity. Daily observations were made throughout the entire trial and there were no apparent symptoms of toxicity in the heifers as a result of feeding these levels of urea.

Additional animals. The two mature dairy type steers fed these rations gained 1.1 lb. per day. The Jersey steer consumed 21.9 lb. of cobs and 9.9 lb.

TABLE 3

Average rumen volatile fatty acid content

Acids	Group A	Group M
	(Molar %)	
Higher acid fraction	0.9	0.9
Valeric	3.2	3.9
Butyric	10.1	10.0
Propionic	18.4	17.0
Acetic	67.4	68.2
Micromoles VFA/milliliter rumen contents	92.05	89.50

of Mix A per day while the Holstein steer consumed 19.5 lb. of cobs and 7.8 lb. of Mix M. No adverse effects were noted.

The average daily gains for the two heifers during the additional 438 days were 0.28 lb. and 0.34 lb. on Rations A and M, respectively. The average daily consumption of cobs was 17.23 and 18.40 lb. per day and of mix was 4.33 and 6.25 lb., in the same order.

The calf from the heifer on Ration A weighed 42 lb. at birth and was very weak and died a few days after. The calf from the heifer on Ration M lived, but weighed only 37 lb. at birth.

The heifer on Ration A produced 3,682 lb. of 4% FCM following parturition during the last 263 days of the extended feeding period and the heifer on Ration M produced 3,594 lb. of 4% FCM following parturition during the last 216 days of the extended feeding period. A single sample of milk from each heifer at mid-lactation contained 16.3% total solids and 6% fat.

The only apparent nutritional inadequacy for these animals maintained on these rations for an extended period of time was energy.

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INFLUENCE OF PRESERVATIVES ON THE FERMENTATION, NUTRIENT RECOVERY, AND FEEDING VALUE OF ALFALFA, STARR MILLET, AND COWPEA AND SUDAN GRASS SILAGES [1]

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SUMMARY

Over a 2-yr. period, 12 silos of silage were used to study the influence of preservatives on silage fermentation, nutrient preservation, and feeding value. Included in the data were silages made from alfalfa, Starr millet, and a mixture of Sudan grass and cowpeas. Preservatives included ground snap corn, citrus pulp, molasses, and sodium metabisulfite. When used with alfalfa, ground snap corn resulted in the most efficient preservation of nutrients followed by citrus pulp, molasses, and sodium metabisulfite. The preservatives resulted in highly significant differences in the rate of fermentation in the silo. Feeding trials, conducted with milking cows and growing heifers, indicated that many differences in silage resulting from preservative and type of fermentation may be partially obscured by grain feeding to producing dairy cows.

In the Southeastern states, interest in alfalfa silage is due primarily to the difficulty in harvesting the first cutting as hay without extensive rain damage. As in other areas, there is general agreement that the use of a preservative greatly increases the chance of successful preservation of the silage, particularly when wilting is not practiced. Despite the use of preservatives, the frequently encountered poor silages and the variability of apparently well-made silages emphasize the lack of understanding of the complex nature of silage fermentation.

The literature on silage research is both extensive and inconclusive. Few experiments are directly comparable to other work. Other workers have used molasses, ground corn, citrus pulp, and sodium metabisulfite with varying degrees of success. Several recent publications have reviewed these factors (1, 2, 3, 5), and there would be little advantage in reviewing them again.

The present experiments were initiated to permit further study of alfalfa silage through the process of fermentation and in feeding trials, with the hope that at least some of the variations in silage could be more accurately measured and understood. In addition to the alfalfa silages, three silages made from grass or mixtures of grass and a legume were included for comparative purposes.

MATERIALS AND METHODS

Silages. The alfalfa silages were all harvested from the same 20-acre field of Oklahoma common alfalfa which was established and maintained by a local farmer in accordance with experiment station recommendations. The Starr

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millet silage was cut from a pure stand at the Station and the Sudan grass and cowpea silage from a mixture of about 80% Sudan grass and 20% cowpeas. All forages were cut with a direct-cut harvester, trucked to the silo, and ensiled immediately. Stage of maturity, date of cutting, and other data are shown in Table 1. When ground snap corn or citrus pulp was used as a preservative, half of the material was added to the load in the field and half placed on top of the load at the silo. Molasses was diluted with water and added from a drum at the silage blower. The sodium metabisulfite was added by hand while the forage was being blown into the silo. All silages were made in 20-ton upright silos. All forage and preservatives were carefully weighed before ensiling and the resulting silage was weighed as fed. Samples of forage for laboratory analysis were collected from each load as it was ensiled. Silage samples were collected on Monday, Wednesday, and Friday during the feeding trials. These samples were frozen and, at the end of the silage feeding period, were composited for analysis. These data are referred to in the tables as the composite of silo. When immediate analysis could not be made, the samples were frozen. The samples used in studying the changes during the fermentation were obtained from 2-in. holes in the two center doors of the silo by the use of a large soil auger. Samples of about 5 lb. were removed at each sampling. This sampling technique can not be expected to accurately represent the entire heterogeneous mass within a silo but, as shown later, it did provide samples indicative of the fermentation proceeding under prevailing conditions in the silo.

Feeding trials. The silos were opened and the silages were group-fed in drylot, one silage at a time, to five milking cows, to secure measures of relative intake and milk production response. The feeding trials lasted for 28 days, during which an average of 20 measurements of intake was recorded. Although the quantity of silage available prohibited longer feeding trials, differences in milk production response are probably accurate enough to measure major differences between silages. Longer feeding trials or the use of change-over

TABLE 1

Stage of maturity, date of cutting, and preservatives used in the silages

Silage No.	Crop	Stage of maturity	Preservative	Cutting date	Comments
1-58 [a]	Alfalfa	Early bud	Snap corn, 200 lb/ton	May 1-2	
1-59	Alfalfa	Early bud	Snap corn, 200 lb/ton	April 17-18	Alternate loads
2-59	Alfalfa	Early bud	Snap corn, 200 lb/ton	April 17-18	
2-58	Alfalfa	Early bud	Citrus pulp, 200 lb/ton	April 29-30	
3-59	Alfalfa	Early bud	Citrus pulp, 200 lb/ton	April 15-16	
3-58	Alfalfa	Early bud	Molasses, 80 lb/ton	April 30-May 1	
4-59	Alfalfa	Early bud	Molasses, 80 lb/ton	April 20-21	Alternate loads
5-59	Alfalfa	Early bud	Molasses, 80 lb/ton	April 20-21	
4-58	Alfalfa	Early bud	$Na_2S_2O_5$, 8 lb/ton	May 2-3	
5-58	Starr millet	10% heads showing	Snap corn, 100 lb/ton	Aug. 25-26	
6-58	Cowpeas and Tift Sudan	Sudan heads showing	Snap corn, 100 lb/ton	Aug. 26-27	
7-58	Cowpeas and Tift Sudan	Sudan heads showing	Snap corn, 200 lb/ton	Aug. 28-29	

[a] Indicates year in which silage was made.

designs to measure animal variability would improve the accuracy of the data and permit smaller differences in milk production to show statistical significance.

Two silages were fed to growing dairy heifers in individual feeding stalls. Twelve heifers were used; intakes of silage were recorded daily and body weights weekly during the seven-day preliminary and 42-day feeding trials. The two silages fed to heifers (2-59 and 5-59) were replicates of Silages 1-59 and 4-59 and were fed to observe the comparative response of growing animals and milking cows to similar silage.

Silage analysis. Dry matter on both the forage and silage was determined by toluene distillation. In 1959, several duplicate samples were also dried in a forced air oven at 70° C. and, on the average, yielded comparable dry matter values. Thus, only the toluene distillation values are reported. Silage pH was determined on a 2-to-1 boiling water extract of fresh silage and the hydrolyzable carbohydrates on dry samples (70° C. for 24 hr.) by the sulfuric acid–phenol technique of Barnett (3). Conventional proximate analyses were done by A.O.A.C. techniques. The volatile acids were liberated from fresh or frozen silage samples and determined, with slight modifications, by the techniques outlined by McCullough and Stasch (6). The total acid values refer to the total acid removed by ether extraction.

RESULTS AND DISCUSSION

Silage fermentation and preservation. The more useful components representing the chemical analysis of the silage as fed are shown in Table 2. With the exception of Silage 4-58 (alfalfa plus sodium metabisulfite), all of the silages would have scored good to excellent on appearance, odor, and stage of maturity at harvesting.

Samples were withdrawn from the silos at intervals and analyzed for several indicators of fermentation. Although all of the silos were studied, in the interest of saving space only selected examples are shown in Table 3. The data presented illustrate the range of conditions observed in the silages. When the

TABLE 2
Chemical composition of the silages as fed

Silage No.	Dry matter	pH	Dry matter basis					
			Crude protein	Crude fiber	Acetic	Propionic	Butyric	Lactic*
	(%)		(%)					
1-58	26	4.3	17.17	25.92	0.94	0.60	0	6.34
1-59	25	4.5	16.58	23.87	3.59	0.44	0.11	11.67
2-59	27	4.3	16.96	24.39	2.71	0.13	0	9.80
2-58	23	4.4	19.87	23.32	1.77	0	0	9.51
3-59	24	4.1	17.78	25.43	2.56	0.28	0	11.15
3-58	21	4.4	19.95	28.03	1.65	1.05	0	12.97
4-59	21	4.7	18.24	31.83	5.96	0.76	0.29	8.12
5-59	21	4.4	18.61	30.20	3.05	0.39	0.79	7.22
4-58	20	5.0	18.96	30.24	0.40	0	0.30	12.40
5-58	22	3.9	11.30	30.17	1.87	0.72	0	12.00
6-58	24	3.9	15.84	26.18	0.92	0.88	0	11.15
7-58	28	4.0	9.80	24.03	1.33	0.67	0	8.09

* Calculated as total acid minus volatile acids.

TABLE 3
Representative changes in the silages during fermentation

Silage	Period after ensiling	pH	Total acid	Dry matter basis				Hydrolyzable carbohydrates
				Acetic	Propionic	Butyric	Lactic	
			(meq/100 g.)	(%)				
4-58	Fresh forage	.....	.....	.....	.....	.....	.....	13.13
	4 days	5.05	66.5	2.49	1.83	0	6.98	7.93
	8 days	5.75	66.4	1.05	0.40	0	8.17	4.75
	12 days	5.80	65.6	1.34	3.13	0	7.85	5.38
	16 days	6.00	70.0	1.72	0.48	0	9.06	4.43
	20 days	5.50	69.0	1.21	0.27	0	10.36	5.42
	30 days	5.45	17.6	0.76	0.17	0	2.34	5.73
	40 days	4.35	46.2	0.92	0.25	0	7.66	5.42
	50 days	5.25	31.2	0.71	0.30	0	3.77	5.12
	60 days	5.35	28.1	0.62	0.24	0	5.01	4.52
	Composite of silo	5.45	71.9	0.40	0	0.30	1.40	4.82
5-58	Fresh forage	.....	.....	.....	.....	.....	.....	30.63
	24 hours	4.45	44.9	0.35	0.47	0	8.20	29.77
	48 hours	3.70	62.0	0.29	1.00	0	9.20	19.34
	72 hours	3.75	69.5	0.44	1.27	0	12.02	21.13
	96 hours	3.55	85.8	0.38	1.17	0	13.60	19.35
	20 days	4.65	96.4	1.09	0.37	0	12.95	19.40
	Composite of silo	4.25	86.2	1.87	0.72	0	12.00	16.45
1-58	Fresh forage	.....	.....	.....	.....	.....	.....	26.56
	4 days	4.45	82.7	0.85	0.91	.....	13.50	18.89
	8 days	4.80	107.4	1.99	1.83	0	12.87	21.67
	12 days	4.90	92.0	2.28	1.61	0	9.28	21.67
	16 days	4.80	100.3	3.61	1.63	0	12.22	16.95
	20 days	5.50	86.2	2.72	0.90	0	11.94	14.74
	30 days	5.65	67.3	1.20	0.40	0	9.15	22.34
	40 days	4.35	65.6	1.03	0.56	0	7.53	21.58
	50 days	3.85	53.2	0.98	0.91	0	7.02	21.30
	60 days	4.00	49.7	0.89	1.64	0	6.08	18.83
	Composite of silo	3.85	42.7	0.94	0.60	0	6.34	22.65
1-59	Fresh forage	.....	.....	.....	.....	.....	.....	28.36
	24 hours	5.30	71.5	1.65	Trace	Trace	3.93	30.87
	48 hours	4.80	121.9	1.79	Trace	Trace	8.20	26.55
	72 hours	4.70	173.0	2.48	Trace	Trace	12.34	19.76
	96 hours	4.70	116.2	1.85	Trace	Trace	7.68	18.06
	20 days	4.50	202.2	3.47	0.10	0.30	12.50	16.37
	Composite of silo	4.30	197.0	3.59	0.44	0.11	11.67	19.23
2-58	Fresh forage	.....	.....	.....	.....	.....	.....	17.18
	4 days	3.95	80.9	1.36	1.99	0	7.71	6.17
	8 days	4.40	89.7	1.46	2.66	0	7.05	7.35
	12 days	4.40	98.1	1.67	1.74	0	10.82	9.20
	16 days	4.20	110.0	2.09	1.75	0	10.90	6.79
	20 days	4.30	92.8	1.50	0.49	0	12.10	6.48
	Composite of silo	4.55	78.7	1.77	0	0	9.51	6.71
3-59	Fresh forage	.....	.....	.....	.....	.....	.....	23.17
	24 hours	5.40	63.0	0.88	0	0	4.74	23.34
	48 hours	5.10	77.6	1.48	Trace	0	4.77	14.02
	72 hours	4.50	111.0	2.09	0.18	0	6.62	11.59
	96 hours	4.40	142.0	2.48	0.13	0	8.84	8.33
	20 days	4.20	146.8	3.27	0.23	0	7.99	7.32
	Composite of silo	4.10	171.0	2.56	0.28	Trace	11.15	3.21

1958 data were examined by analysis of variance, significant differences were found between silages for only the propionic and lactic acids. The absence of differences between days for all items indicated that the sampling intervals of four days were missing the major changes in fermentation. Beginning with

the summer silages in 1958, and including the 1959 alfalfa silages, sampling was done at 24-hr. intervals for the first four days and on the 20th day. For Silages 5-58, 6-58, and 7-58 there were significant differences between days for acetic and lactic acids. The 1959 alfalfa silages also showed significant differences for acetic and lactic acids due to days. The data for both years showed the trends characteristic (5) of silage fermentation. Acetic acid levels increased in early stages and then decreased while lactic acid levels exhibited a tendency to increase steadily. Propionic acid usually appeared in the early stages and then disappeared. All acids exhibited irregular curves, which may be due to sampling errors or actual changes due to breakdown and build-up of acids in fermentation.

Silages 4-58 (alfalfa plus sodium metabisulfite) was the only silage that showed little tendency for the fermentation to be complete even at 60 days after ensiling (Table 3). This silage showed indications of acids being used as energy for the microorganisms, the heavy loss in crude fiber (Table 4) indicated a breakdown of complex carbohydrates, and the general failure of the pH to drop despite the buildup of acids would indicate that the buffer capacity was not easily overcome. The 38% loss of dry matter certainly indicates the potential loss of silage nutrients in this type of fermentation. The relatively small amount of butyric acid is difficult to explain in light of the 56% loss of crude protein. The silage had an offensive odor which was thought to indicate amines, since it was largely the odor of decaying fish.

Silage 1-58 and 1-59 (alfalfa plus snap corn) represented a contrast in fermentation of silages supposedly made in the same manner. The 1958 silage required 40 days to reach a fermentation stage with a pH of 4.3 and 50 days for a condition similar to the final silo composite. The 1959 silage was a more direct fermentation, with a 4% loss of dry matter in contrast to the 13% loss in 1958. The available data do not offer an explanation. In light of the recent findings of Gordon *et al.* (4) and Wieringa (7) on the influence of chopping and the degree of laceration on silage fermentation and dry matter preserva-

TABLE 4
Preservation of ensiled nutrients

Silage No.	Silage	Crude protein	Ether extract	Crude fiber	NFE	Ash	Hydrolyzable carbohydrates	Dry matter
					(%)			
1-58	Alfalfa and snap corn	75.0	162.5	98.1	88.7	66.6	75.4	88.5
1-59		82.7	233.3	114.2	88.5	100.0	65.2	96.6
2-59		80.7	181.8	114.1	85.6	91.6	49.1	93.6
2-58	Alfalfa and citrus pulp	85.5	148.4	87.5	84.9	85.6	34.1	86.7
3-59		74.7	153.3	97.0	71.8	76.2	10.9	80.9
3-58	Alfalfa and molasses	70.4	141.7	89.6	67.4	71.1	21.5	75.5
4-59		72.1	171.4	110.0	76.8	82.1	17.9	87.4
5-59		75.0	216.6	107.0	83.6	83.9	14.9	90.4
4-58	Alfalfa, $Na_2S_2O_5$	54.0	108.0	72.0	58.3	60.5	22.9	62.5
5-58	S. millet, snap corn	86.7	116.8	102.7	79.0	86.3	46.9	87.4
6-58	Sudan, snap corn	100.0	126.4	94.3	90.6	96.9	58.2	94.4
7-58	Sudan, snap corn	92.6	125.0	85.9	83.7	85.3	58.7	86.1

tion, and from observations made at the time of ensiling, a possible explanation for the differences may be postulated. The 1958 silage was harvested after 40 tons of forage had been cut with the machine without changing blades. At this time there was a definite tendency for the degree of chopping to decrease. The 1959 silage was harvested with a new and more powerful harvester which definitely gave better performance. Thus, the difference in preservation of 9% may be due to a poor management decision in not keeping the forage harvester in top operating condition. Silage 5-58 (Starr millet plus snap corn) is an illustration of a low-protein, lightly buffered silage that reached a satisfactory fermentation in 24 to 48 hr., and represents a contrast to the difficulties encountered with the alfalfa silages.

Silages 2-58 and 3-59 (alfalfa plus citrus pulp) illustrate two silages which apparently exhibited rapid and satisfactory fermentation pictures. The fermentations resulted in 15 and 19% losses in dry matter. The 66 and 90% losses of hydrolyzable carbohydrates and the total acidities of 78.7 and 171.0 meq/100 g. indicate a very rapid production of acid but a slow drop in pH. Thus, a rapid and violent fermentation may give a picture of an efficient fermentation, but in reality it is not as desirable as the slower but more efficient fermentation of the silages made with snap corn.

Since the primary change in nutrients during fermentation is the conversion of carbohydrates to acids, a general measure of this conversion is the ratio of the recovered acid to carbohydrate lost. The average ratios for the silages were: alfalfa and snap corn, 1.16 lb. acid per pound of hydrolyzable carbohydrates lost; alfalfa and citrus pulp, 0.80; alfalfa and molasses, 0.71 and, the millet and Sudan grass silages, 0.81.

Feeding trials. The data collected during the feeding trials are shown in Table 5. The trials are best considered as intake trials, but the production data are presented despite their limited value. With the exception of Silage 4-58 (alfalfa plus sodium metabisulfite), all of the silages were readily consumed by the cows. Silage 4-58 had such an objectionable odor that it was fed to dry

TABLE 5

Dry matter intake, ratio of grain fed, level of production, and 28-day response of the cows fed the silages

Silage No.	Silage intake			Ratio of grain: milk	Initial milk production	28-day change in	
	As fed	d.m.	d.m/ 1,000 lb.			body wt.	4% FCM
	(lb.)				*(lb. FCM)*	*(lb.)*	*(%)*
1-58	84	21.7	22.6	1: 6	24.0	−	− 1.9
1-59	92	23.3	23.8	1: 10	27.2	−	−15.9
2-59	Fed to dairy heifers						
2-58	124	28.6	27.0	1: 6	23.9	+	+10.7
3-59	91	21.8	22.5	1: 10	27.2	+	+ 8.4
3-58	116	23.9	23.0	1: 4	26.4	+	−14.5
4-59	84	17.9	19.2	1: 4	27.1	+	− 1.0
5-59	Fed to dairy heifers						
4-58	73	14.7	15.4	Not suitable for milk production			
5-58	110	24.8	25.5	1: 4	22.3	+	+ 3.7
6-58	115	27.4	28.9	1: 4	22.6	+	+ 5.0
7-58	101	27.9	29.1	1: 4	22.9	+	+12.0

cows to obtain relative intake. The data demonstrate that silages with large differences in type of fermentation and efficiencies of preservation may yield similar results in feeding trials. The equating factor in such observations may be the grain supplementation used in the feeding trials. The two silages fed to dairy heifers may illustrate something of this relationship. When the heifers were fed silage alone, those receiving Silage 2-59 (alfalfa plus snap corn) had an average daily gain of 1.68 lb. and required 670 lb. dry matter per 100 lb. gain; whereas, those receiving 5-59 (alfalfa plus molasses) gained 1.34 lb. and used 880 lb. dry matter per 100 lb. of gain. When grain was fed to supply 50% of the calculated TDN required for maintenance, the respective average daily gains were 1.95 and 1.88 lb. and the dry matter required per 100 lb. of gain was 581 and 597. The vast improvement in average daily gain and feed efficiency of the 5-59 silage with grain feeding may indicate something of the equalizing effects of grain feeding.

The two ratios of grain to milk fed with the snap corn and citrus pulp silages were used to obtain data relative to the level of grain feeding required with silages containing feedstuffs used as preservatives. The excellent production obtained with a grain-to-milk ratio of 1:6, and the variable results with a ratio of 1:10, would probably render a general recommendation of 1:8 as a satisfactory rate of feeding in practice.

Since 28-day weights are highly variable, the changes in weight are indicated as plus or minus for the group. In general, all of the silages except 4–58 provided a satisfactory ration for milk production. The data do indicate that a knowledge of the preservative used, and its over-all influence on nutrient preservation, is necessary to determine grain feeding levels and the extent to which measures such as hay feeding may be necessary to control body weight losses.

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EVIDENCE CONCERNING THE ORIGIN OF ANDROGENIC ACTIVITY IN FEMALE BOVINE FECES

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SUMMARY

An investigation has been conducted to study the source and formation of the androgenic activity of the incubated cow feces. The results indicate that the androgenic activity is dependent upon the presence of two factors: (1) an androgen precursor which is present only in cow feces, and (2) microbial activity which is present both in bull and in cow feces. Apparently, progesterone can serve as a precursor of the androgenic substance.

In 1942, while studying the feasibility of using cow manure as a source of B vitamins for chickens, Hammond (5) discovered androgenic activity in dried cow feces. A later report by Riley and Hammond (11) showed androgenic activity in cow feces but not bull feces, after 24-hr. incubation at about 45° C. Feces incubated at 80° C. for 48 hr. exhibited no activity. The active factor did not appear to be of dietary origin. Turner (12) demonstrated that 45° C. was an optimum temperature to dry fresh cow manure for maximum androgenic activity. Manure incubated at temperatures greater than 45° C. declined in androgenic activity. Slow drying at temperatures lower than 45° C. reduced the potency of incubated manure. Fresh feces did not produce an androgenic response. Turner (13) found that the androgenic activity was not destroyed when cow feces were autoclaved (sterilized) and subsequently dried at 45° C. Miller and Turner (8) believed that neither enzymic nor bacterial action was responsible for the androgenic activity of incubated female bovine feces. Marlow (7) also drew this same conclusion after getting negative results from incubating wet cow feces in fermentation jars for a week.

Although others dismissed the possibility of bacterial action being responsible for the androgenic activity of cow feces, evidence substantiating this point seemed deserving of further study. Therefore, a series of trials was conducted to determine if microorganisms might have a role in the androgenic response found in cow feces. Also, it was desired to gain information which might elucidate the mechanism involved in the release of male hormone from the feces of the female bovine.

EXPERIMENTAL METHODS

A chick comb growth assay method similar to that used by Riley and Hammond (11) was employed to measure the relative androgen content of the various fecal samples. Cow feces were collected from cows in the eighth month

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of pregnancy, as there was evidence that fecal androgen activity was highest at this period (4). Manure was dried at 45° C. for approximately 48 hr. Selected samples of manure were autoclaved at 15 p.s.i. for 25 min. Inoculation of sterilized feces was accomplished by adding an amount of fresh cow feces equal to 5% of the weight of the sterilized material. For each trial dried fecal material was ground and added to the experimental ration so that this feed contained 10% cow feces. Statistically significant differences were determined by the use of the Duncan Multiple Range Test (1, 6). For each trial a control group of chicks was fed the basal chick starter ration which contained no cow feces.

RESULTS AND DISCUSSION

In the first experiment, a series of trials was completed to determine whether or not microbial action was responsible for the androgenic activity found in incubated cow feces. The results of three trials in Table 1 show the effects of

TABLE 1

Effect of sterilization and inoculation of cow feces upon the androgenic response measured by the chick comb growth assay

Trial	Average No. chicks per group	Average comb weights of chicks *(mg.)*			
		Control (feed only)	Sterilized	Sterilized and inoculated	Regular incubation
I	18	186	182	422	538
II	15	230	230	370[a]	440
III	20	95	70	129	188

Figures underlined by the same line are not significantly different.
[a] Inoculated with fresh bull feces.

sterilization or of sterilization and inoculation upon the androgen content of dried cow feces. In each instance chicks receiving sterilized cow feces had combs comparable to those of chicks receiving the basal ration. Contrary to Turner's results (13), sterilized cow feces were found to be devoid of androgenic activity. When sterilized cow feces were inoculated with fresh cow feces and then incubated, an androgenic response was obtained. This indicates that microbial activity is essential for the appearance of androgenic activity. In each trial cow feces which were dried but not sterilized produced a greater response than feces which had been sterilized and inoculated. This greater response may have been due to partial destruction of the active factor during autoclaving or poor distribution of the inoculating material. When sterilized cow feces were inoculated with a small amount of fresh bull feces, and then incubated, an androgenic response was obtained. Thus, it seemed that cow feces contained a precursor and microflora capable of producing an androgenic effect, whereas bull feces contained the same microflora but not the necessary precursor. This experiment further suggests that microbial activity is responsible for the androgenic factor in dried feces of the female bovine.

A second experiment was conducted to determine if progesterone might serve as the precursor of the androgenic factor. Progesterone was considered

a likely precursor, because its formula is very similar to that of some androgens, and because it is present in the female and not in the male. Also, it has been shown that female androgen excretion occurs during the normal estrus cycle with a peak coincident with corpus luteum maturation (13). This hypothesis was tested by adding progesterone to feces prior to incubation. Preliminary trials gave some indication that addition of progesterone to fecal samples before drying increased the androgenic content. While these experiments were in progress, a report by Miller and Turner (9) was published which showed that the injection of bulls and heifers with progesterone caused a great increase in the fecal androgen content. The androgenic activity is not due to progesterone per se, as we found that the addition of 500 mg. of progesterone per kilogram of feed caused a decrease in comb weights. This is in agreement with the findings of Miller and Turner (9), who reported lower comb weights when 80 or 160 mg. of progesterone were added per kilogram of feed.

The addition of increasing amounts of progesterone to cow feces prior to the incubation period caused an increased androgenic response in chicks (Table 2). The maximum response was obtained when 385 mg. of progesterone were

TABLE 2

Effect of adding increased amounts of progesterone to cow feces before incubation

Group	C	1	2	3	4	5	6	7
No. chicks per group	14	14	14	14	14	11	14	13
Milligrams progesterone added per kilogram of feed	f[a]	0	7.6	30.8	61.6	123.2	246.4	385
Average chick comb weight *(mg.)*	216	259	276	316	339	404	489	565

Figures underlined by the same line are not significantly different.
[a] f ration contained no feces and no progesterone.

added to feces per kilogram of feed. Thus, it seems that progesterone can serve as a precursor of the androgenic factor found in dried cow feces. To further test this hypothesis, progesterone was added to bull feces prior to incubation. The results presented in Table 3 are not as consistent as those obtained with

TABLE 3

Effect of adding increased amounts of progesterone to bull feces before incubation

Group	C	3	2	4	1	5	7	6
No. chicks per group	14	15	13	14	14	14	13	14
Progesterone added kilogram feed	f[a]	20.5	51.1	41.0	0	82.0	257.0	164.0
Chick comb weight	216	261	266	307	332	363	377	405

Figures underlined by the same line are not significantly different.
[a] Feces but no progesterone.

cow feces; however, increased androgenic response was shown to occur when 82, 164, or 257 mg. of progesterone were added in the feces per kilogram of feed. The relatively lower response obtained with chicks of Group Seven might be attributed to incomplete mixing of the progesterone in the feces. The levels

of progesterone added to the bull feces were considerably lower than the amounts added to the cow feces, as the amounts of progesterone were added to a standard amount of wet feces and it happened that the cow feces contained less dry matter. The total progesterone content of the cow feces was considerably greater than that of the bull feces, since it contained naturally excreted progesterone as well as the added progesterone.

From our results it seems that the androgenic response found in dried cow feces is the result of progesterone being transformed to an androgenic substance by microflora (after fecal excretion) during the drying period. Thus, bull feces appear to be inactive due to the absence of a precursor such as progesterone. Fresh cow feces are inactive, since an incubation period is necessary for microorganisms to convert the progesterone to an androgen. Turner's (13) temperature work indicates that 45° C. may be the optimum temperature for the microbial bacterial action and that temperatures above 80° C. prevent their activity. The fact that androgenic activity is not destroyed when cow feces are incubated at 45° C. and later heated at 80° C. indicates that microbial action took place at the lower temperature and that the higher temperature did not destroy the androgenic substance. The microorganisms may be aerobic, since incubating wet cow feces produced no androgenic activity.

Our data do not prove that progesterone is the natural precursor, but such a hypothesis is supported by the work of Vischer and Wettstein (14), who have shown that *Fusarium solani* and *Fusarium caucasiucum* degrade progesterone to △ 1,4-androstadiene-3,17-dione in sizable quantities. Fried *et al.* (2) have demonstrated that the fermentation of progesterone with *Streptomyces lavendulae* yields △ 1,4-androstadiene-3,17-dione and △ 1,4-androstadiene-17-ol-3-one. The component androgens of cow feces have been identified (10) and it was shown that △ 1,4-androstadiene-3,17-dione is the major component. Thus, it would appear that the progesterone in female bovine feces is converted during the drying process to a product which exhibits androgenic activity.

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SUCCESSFUL DIHYDROTACHYSTEROL (A.T. 10) REPLACEMENT THERAPY IN LACTATING THYRO-PARATHYROIDECTOMIZED RATS [1]

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SUMMARY

Thyro-parathyroidectomy (TPEC) was performed in ten albino rats on Day 4, in 66 albino rats on Day 7 of lactation. Replacement therapy was only partially successful when 187.5 μg. dihydrotachysterol (A.T. 10)/day was injected subcutaneously from Days 5-13. Completely successful replacement therapy was achieved when 60, 80, and 100 μg. A.T. 10/100 g. body weight/day were given orally from Days 7-13 of lactation. All animals received daily 3.5 and 3 μg. thyroxine (T4)/100 g. per day, respectively, from Day 4 or 7 until end of experiment. Criteria for lactational performance were prenursing litter weight and milk yield on Days 14 and 15-21. Nursing was standardized with 1 U.S.P. unit oxytocin (OXT), injected subcutaneously from Days 14-21 immediately before each nursing. Discontinuation of A.T. 10 in TPEC lactating rats on Day 13 resulted in fatal attacks of tetany in 35 of 66 of the superior lactating rats between Days 16 and 21. In 11 rats nonfatal attacks of tetany, combined with reduced lactational performance, occurred during the same period. Eleven other rats not observed to show signs of tetany were believed to have been completely parathyroidectomized. It is suggested that lower losses of serum Ca due to poor lactational performance (as indicated by depressed litter weight and milk yield on Day 21) were responsible for the absence of tetany. Nine rats with lactational performance until Day 21 similar to intact control rats were considered to have accessory parathyroid glands. Rats successfully TPEC lost body weight, whereas intact controls gained slightly.

It has been shown that parathyroid hormone (PTH) plays an important role in normal lactation (8). Completely successful replacement therapy in thyro-parathyroidectomized (TPEC) lactating rats was achieved recently (9). This was made possible by administration of optimal amounts of thyroxine (T4) as well as PTH. Based on thyroxine secretion rate determinations of lactating rats and on studies concerning galactopoietic efforts of subcutaneously injected T4, 3 μg/100 g/day exerted positive effects on lactational performance when given from Days 7-13 under standardized experimental conditions in strain of rats used (3, 5). Amount of PTH administered concurrently with T4, in order to maintain better than normal lactation in TPEC rats, was found to be 29-30 U.S.P. units/100 g/day when given in two daily injections of 2 × 40 U.S.P. units (9).

However, PTH is not readily available in large amounts, has a short half-life, and must be injected. A compound produced by the irradiation of plant

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TABLE 1

Replacement therapy with thyroxine and subcutaneously administered dihydrotachysterol (A.T. 10) in thyro-parathyroidectomized lactating rats

Daily replacement therapy [a] Days 7-13	Rats	Mother's avg. body weight			Average litter weight			Milk yield Day 14	
		Day 4	Day 14 Post-nursing	Gain or loss	Day 4	Day 14 Pre-nursing	Gain	Total	Total as % of post-nursing litter wt.
	(No.)	*(g.)*	*(g.)*	*(%)*	*(g.)*	*(g.)*	*(%)*	*(g.)*	
Intact controls,[b] no treatment	30		300			166		6.2	3.8 ± .3
Intact controls,[c] T4: 3 μg/100 g., A.T. 10: none	30		286			166		9.5	5.2 ± .2
TPEC: Day 4,[d] A.T. 10: none, T4: 3.5 μg/100 g.	8	271	240	−11 ± 3	56	93	67 ± 16	1.6 ± .4	1.7 ± .3
TPEC: Day 4, μg. A.T. 10: 187.5/(subc.), T4: 3.5 μg/100 g.	7	283	266	− 2.3 ± 1.6	57 ± 1.6	122 ± 3.6	117 ± 7	3.6 ± .4	2.8 ± .4
TPEC: Day 4, A.T. 10: 187.5 μg. (subc.), T4: 3.5 μg/100 g. Presumably accessory parathyroid tissue	3	295	295	± 0	56	166	198	7.9	4.6

A.T. 10 = dihydrotachysterol; T4 = thyroxine; TPEC = thyro-parathyroidectomy.

[a] All rats, thyro-parathyroidectomized on Day 4, received 125 μg. A.T. 10 on Day 3 if A.T. 10 replacement therapy was given from Days 4-13.

[b] Data from Grosvenor and Turner (4).

[c] Data from Grosvenor and Turner (5).

[d] Data from von Berswordt-Wallrabe and Turner (8).

sterols called dihydrotachysterol (A.T. 10, or Hytakerol),[3] which is effective orally, has been known to have physiological properties similar to the hormone of the parathyroid gland. This compound could be produced in large amounts for use with dairy cattle in the prevention of parturient paresis (milk fever) if it were shown to completely replace PTH in TPEC lactating animals.

Purpose of this study was to determine if graded levels of orally administered A.T. 10, together with optimal amounts of T4, would maintain intense lactation in TPEC rats successfully under standardized conditions, based on litter weight and milk yield determinations.

MATERIALS AND METHODS

Primiparous lactating rats of Sprague-Dawley-Rolfsmeyer strain were housed in individual cages, fed Purina Lab. Chow and water ad libitum. The animal room was maintained at a uniform temperature of 78 ± 1° F. Shortly after delivery each litter was reduced to six young.

Animals thyro-parathyroidectomized on Day 4 were operated and handled as described previously (8). On Day 3, 125 mg. dihydrotachysterol (AT 10) were given subcutaneously. Daily replacement therapy was started shortly after operation: 3.5 mg. thyroxine/100 g. B.W. plus 187.5 μg. AT 10 subcutaneously (Table 1). Milk secretion was measured as described previously (8). Animals thyro-parathyroidectomized on Day 7 were operated in less than 20 min. under ether anesthesia. On Day 5: 20 μg. AT 10/100 g. body weight (B.W.), on Day 6: 40 μg. AT 10/100 g. B.W. were given orally by stomach tube. Shortly after operation, replacement therapy was started: 3 μg. thyroxine/100 g. B.W/day during experiment until Day 21, plus graded amounts of orally administered AT 10 until Day 13 (Table 2). In animals operated on Day 7, nursing was standardized with 1 U.S.P. unit oxytocin (OXT).[4] Parameters of lactational performance were mean litter weight and milk yield. Amount of milk removed by litter of six was estimated by the standardized method described previously (2). In order to repeat this test at 24-hr. intervals, litters were weighed at the end of 10 hr. of isolation period, immediately replaced and allowed to nurse for 30 min. Litters were weighed again to the nearest 0.5 g. Difference in litter B.W. before and after each nursing was used to represent amount of milk secreted during previous 10 hr. After this test litters were allowed to stay overnight with mothers. To prevent feed intake other than mother's milk by young, mothers were allowed to eat only during 10-hr. separation period from Day 15 on (Table 6).

To keep litter size uniform, dead young were replaced by healthy young of same age and size. A.T. 10 was injected subcutaneously in animals operated on Day 4; given orally by stomach tube in animals operated on Day 7. Last replacement therapy was given in both cases in the afternoon of Day 13.

[3] Kindly supplied by Sterling-Winthrop Research Institute, Rensselaer, New York. The commercial solution of A.T. 10, or dihydrotachysterol (Hytakerol), contains about 1,250 μg. of active principle/milliliters. Hytakerol is the registered trademark of Winthrop Chemical Co.

[4] Oxytocin kindly supplied by Armour Laboratories, Chicago, Illinois.

TABLE 2

Replacement therapy with thyroxine and graded levels of orally administered dihydrotachysterol (A.T. 10) in thyro-parathyroidectomized lactating rats

Daily replacement therapy [a] Dihydrotachysterol (A.T. 10) Days 7-13 (orally)	Rats	Average body weight of mothers			Average litter weight			Average milk yield			
		Day 6	Day 14 Post-nursing	Gain or loss	Day 6	Day 14 Pre-nursing	Gain	Day 14 Total	Day 14 Total as % of post-nursing litter wt.	Day 15 Total	Day 15 Total as % of post-nursing litter wt.
	(No.)	*(g.)*	*(g.)*	*(%)*	*(g.)*	*(g.)*	*(%)*	*(g.)*		*(g.)*	
Intact controls,[b] A.T. 10: none, T4: none	38	271	277	2.3 ± .6	70	153	120 ± 6	9.9 ± .3	6.0 ± .1		
Intact controls, A.T. 10: none	21	286	296	3.6 ± .6	76 ± 2^{1}	167 ± 4^{4}	124 ± 5^{5}	$11.3 \pm .5^{13}$	$6.3 \pm .2^{18}$	$12.3 \pm .4^{23}$	$6.8 \pm .3^{30}$
TPEC: Day 7, A.T. 10: 60 μg/100 g. Affected with tetany [c]	13	293	286	−2.0 ± 1.6	72 ± 2	154 ± 5^{5}	115 ± 3^{9}	$10.0 \pm .6^{14}$	$6.1 \pm .3^{19}$	$11.8 \pm .7^{24}$	$7.1 \pm .4^{30}$
TPEC: Day 7, A.T.: idem., No visible signs of tetany	10	289	288	− .2 ± 1.0	71 ± 2	151 ± 5^{6}	113 ± 5^{10}	8.6 ± 1.0^{15}	$5.3 \pm .5^{20}$	$11.1 \pm .7^{25}$	$6.9 \pm .4^{30}$
TPEC: Day 7, A.T. 10: 80 μg/100 g. Affected with tetany [c]	15	267	250	−6.0 ± 1.0	81 ± 2^{2}	159 ± 4^{7}	97 ± 4^{11}	$9.9 \pm .5^{16}$	$5.9 \pm .3^{21}$	$12.3 \pm .4^{26}$	$7.1 \pm .2^{31}$

TABLE 2 (concluded)

Replacement therapy with thyroxine and graded levels of orally administered dihydrotachysterol (A.T. 10) in thyro-parathyroidectomized lactating rats

Daily replacement therapy [a] Dihydrotachysterol (A.T. 10) Days 7-13 (orally)	Rats	Average body weight of mothers			Average litter weight			Average milk yield			
		Day 6	Day 14 Post-nursing	Gain or loss	Day 6	Day 14 Pre-nursing	Gain	Day 14 Total	Day 14 Total as % of post-nursing litter wt.	Day 15 Total	Day 15 Total as % of post-nursing litter wt.
	(No.)	*(g.)*	*(g.)*	*(%)*	*(g.)*	*(g.)*	*(%)*	*(g.)*		*(g.)*	
TPEC: Day 7, A.T. 10: idem., No visible signs of tetany	6	261	247	−5.2	71	138	96	8.8	6.0	10.0	6.7
TPEC: Day 7, A.T. 10: 100 μg/100 g. Affected with tetany [c]	18	296	284	$-3.8 \pm .9$	82 ± 2^{a}	169 ± 6	107 ± 4^{12}	$9.3 \pm .8^{17}$	$5.1 \pm .4^{22}$	$12.0 \pm .8^{27}$	$6.5 \pm .3^{32}$
TPEC: Day 7, A.T. 10: idem., No visible signs of tetany	4	299	289	−3.1	79	164	108	9.5	5.5	11.5	6.5

Students "t" Test

1-2	$.1 < p < .2$	4-6	$.01 < p < .025$	8-11	$< .001$	18-20	$.025 < p < .05$	15-25	$.05 < p < .1$	19-29	$.05 < p < .1$
1-3	$.005 < p < .01$	4-7	$.1 < p < .2$	8-12	$.01 < p < .025$	18-22	$.01 < p < .025$	16-26	$.001 < p < .005$	20-30	$.01 < p < .025$
4-5	$.05 < p < .1$	8-9	$.2 < p < .4$	13-15	$.01 < p < .025$	13-23	$.1 < p < .2$	17-27	$.01 < p < .025$	21-31	$.001 < p < .005$
		8-10	$.1 < p < .2$	13-17	$.025 < p < .05$	14-24	$.05 < p < .1$	18-28	$.2 < p < .4$	22-32	$.005 < p < .01$

A.T. 10 = dihydrotachysterol; OXT = oxytocin; T4 = thyroxine; TPEC = thyro-parathyroidectomy.

[a] All rats (if not otherwise indicated) received 3 μg. thyroxine/100 g. body weight/day from Day 7-21 of lactation. Nursing was standardized with 1 USP unit oxytocin given subcutaneously. All rats thyro-parathyroidectomized on Day 7 received on Day 5: 20 μg. A.T. 10/100 g., on Day 6: 40 μg. A.T.10/100 g. body weight orally by stomach tube.

[b] Data from von Berswordt-Wallrabe, Moon, and Turner (10).

[c] See Table 3.

TABLE 3

Incidence of tetany in thyro-parathyroidectomized lactating rats after withdrawal of subcutaneous injected parathormone and orally administered A.T. 10 replacement therapy, respectively

Daily replacement therapy [a] PTH: Days 7-13, 14 A.T. 10: Days 7-13	Rats	Final replacement for parathyroids was given on Day PP	Days postpartum									Total of affected rats [b]		Total of rats without visible signs of tetany
			13	14	15	16	17	18	19	20	21	t	T	
	(No.)													
Intact controls, PTH: none, A.T. 10: none	15													15
TPEC: Day 7, PTH: 2 × 40 U.S.P. units [c]	12	13	1T	9T									10	2
TPEC: Day 7, PTH: idem. [c]	22	14				1t 11T	1t 2T					2	13	7
TPEC: Day 7, A.T. 10: 60 μg/100 g. (orally)	23	13					(1)1t 2T	3t 3T	2t	1t	1t	8	5	10
TPEC: Day 7, A.T. 10: 80 μg/100 g. (orally)	21	13				(1)t 1T	1t 4T	(1)t 4T	2T	3T		1	14	6
TPEC: Day 7, A.T. 10:100 μg/100 g. (orally)	22	13					2 t 2T	(2)t 2 T	(2)t 7T	4T	1T	2	16	4

A.T. = dihydrotachysterol; PTH = parathyroid hormone; T4 = thyroxine; T = fatal attack of tetany; t = nonfatal attack of tetany; figures in parentheses: nonfatal attacks of tetany of animals which later died with tetany.

[a] All rats received 3 μg. thyroxine/100 g. body weight/day from Days 7-21 of lactation. All A.T. 10–treated animals, thyro-parathyroidectomized on Day 7, received on Day 5: 20 μg. A.T/100 g., on Day 6: 40 μg. A.T. 10/100 g. body weight orally by stomach tube.

[b] Only first nonfatal attack of affected animal recorded.

[c] Data from von Berswordt-Wallrabe and Turner (9).

RESULTS

Thyro-parathyroidectomized rats operated on Day 4, given replacement therapy of 3.5 μg. T4/100 g/day and 187.5 μg. A.T. 10/day subcutaneously from Day 7-13 failed to raise litters of six normally, although mother's B.W. loss, prenursing litter weight, and lactational performance at Day 14 were alleviated as compared to TPEC rats given no replacement therapy for parathyroids (Table 1).

In contrast herewith, TPEC rats operated on Day 7, given replacement therapy of 3 μg. T4/100 g/day and 60, 80, and 100 μg. A.T. 10/100 g/day, respectively, from Day 7-13, raised litters of six young successfully, reaching normal litter weights, although litter weight gain from Days 6-14 was lower because of high litter weights on Day 6 (Table 2).

Lactational performance of completely TPEC rats was slightly depressed on Day 14 as compared to intact controls given the same amount of T4. On Day 15, however, milk yield was markedly increased in all TPEC rats over Day 14, reaching levels of control animals (Tables 2, 4).

To determine completeness of operation, A.T. 10 was discontinued on Day 13. Milk yield test was repeated daily until Day 21. Thyroid replacement was given daily during this period to assure high milk yields. During this test period from Days 16-21, 35 TPEC rats died with tetany. Eleven rats showed repeatedly nonfatal attacks of tetany concurrently with significantly reduced lactational performance (Table 3). Eleven animals had poor lactational performance and practically no litter weight increase, although they did not exhibit visible symptoms of tetany. They were tentatively considered as successfully operated and without accessory parathyroid tissues. Finally, a group of nine rats showed steady litter weight increases combined with normal lactational performance. Consequently, they were tentatively considered to possess accessory parathyroid tissue.

Regrouping of all TPEC ratss according to these criteria, as shown in Table 4, proves that rats which died in tetany were the best lactators, slightly better than intact controls under same standardized conditions, particularly on Days 15 and 16 (Table 5). Seven excellent lactators had nonfatal attacks of tetany. Subsequently, their milk yield declined markedly, but upon returning to normal they had fatal attacks of tetany. This explains why the average of this group was depressed on Days 17-19 in comparison to the controls (Tables 3, 4, 5).

Mother rats became familiar with the technique of restricted feeding hours immediately and ate huge amounts of pellets during the 10-hr. isolation period (Table 6). Therefore, in control rats and rats with presumably accessory parathyroid tissues, body weight was not affected adversely on Day 21. TPEC rats generally lost weight even under normal feeding conditions (Table 2).

Blood calcium determinations supported regrouping of TPEC rats from Days 14-21. Although absolute figures are unusually high, animals with nonfatal attacks of tetany, as well as those which presumably had been operated suc-

TABLE 4

Prenursing litter weight, lactational performance, and blood calcium levels of thyro-parathyroidectomized rats after withdrawal of orally administered A.T. 10 replacement therapy on Day 13 of lactation

Daily treatment for all rats T4: 3 μg/100 g. OXT: 1 U.S.P. unit	Rats	Day 14			Day 15			Day 21			(Prenursing) Litter weight gain between Days 14 and 21	Blood calcium [a] on Day 21 (postnursing)
		Prenursing litter wt.	Milk yield		Prenursing litter wt.	Milk yield		Prenursing litter wt.	Milk yield			
			Total	Total as % of postnursing litter wt.		Total	Total as % of postnursing litter wt.		Total	Total as % of postnursing litter wt.		
	(No.)	*(g.)*	*(g.)*		*(g.)*	*(g.)*		*(g.)*	*(g.)*		*(%)*	*(mg %)*
Intact controls	15	163 ± 4^{1}	$10.9 \pm .6^{4}$	$6.3 \pm .3^{9}$	164 ± 4^{14}	$12.1 \pm .6^{17}$	$6.9 \pm .4^{22}$	198 ± 5^{27}	19.0 ± 1.2^{31}	$8.7 \pm .4^{35}$	22 ± 2^{39}	(14) $12.8 \pm .2^{42}$
TPEC: Day 7, presumably accessory parathyroid tissue	9	156 ± 7	$9.8 \pm .4^{5}$	$5.9 \pm .2^{10}$	158 ± 7	11.2 ± 1.0^{18}	$6.6 \pm .4^{23}$	189 ± 9^{28}	16.6 ± 1.5^{32}	$8.0 \pm .4^{36}$	21 ± 4	(6) $12.7 \pm .4$
TPEC: Day 7, died with tetany	35	165 ± 3	$9.6 \pm .5^{6}$	$5.5 \pm .2^{11}$	167 ± 3	$12.3 \pm .5^{19}$	$6.8 \pm .2^{24}$					
TPEC: Day 7, Nonfatal attack of tetany	11	151 ± 11^{2}	$10.1 \pm .7^{7}$	$6.2 \pm .3^{12}$	153 ± 7^{15}	$11.6 \pm .6^{20}$	$7.1 \pm .3^{25}$	156 ± 7^{29}	10.2 ± 1.6^{33}	$6.0 \pm .7^{37}$	3 ± 3^{40}	(9) $9.1 \pm .3^{43}$
TPEC: Day 7, no visible signs of tetany but presumably no accessory parathyroid tissues	11	144 ± 6^{3}	$8.1 \pm .9^{8}$	$5.2 \pm .5^{13}$	143 ± 6^{16}	$10.7 \pm .6^{21}$	$7.1 \pm .5^{26}$	(10) 150 ± 6^{30}	(10) $10.4 \pm .5^{34}$	(10) $6.5 \pm .2^{38}$	(10) 4 ± 2^{41}	$9.4 \pm .5^{44}$

A.T. 10 = dihydrotachysterol; OXT = oxytocin; T4 = thyroxine; TPEC = thyro-parathyroidectomy; figures in parentheses: number of rats.

[a] Calcium determinations by Mr. David Henschel.

Students "t" Test

1-2	$.1 < p < .2$	17-21	$.1 < p < .2$	42-43, 44	$< .001$
1-3	$.005 < p < .01$	27-28	$.2 < p < .4$	4-17	$.1 < p < .2$
4-6	$.05 < p < .1$	27-29, 30	$< .001$	5-18	$.1 < p < .2$
4-8	$.001 < p < .005$	31-32	$.1 < p < .2$	6-19	$< .001$
9-11	$.025 < p < .05$	31-33, 34	$< .001$	7-20	$.1 < p < .2$
9-13	$.01 < p < .025$	35-36	$.1 < p < .2$	8-21	$.01 < p < .025$
14-15	$.1 < p < .2$	35-37, 38	$< .001$	9-22	$.1 < p < .2$
14-16	$.001 < p < .005$	39-40, 41	$< .001$	10-23	$.1 < p < .2$
				11-24	$< .001$
				12-25	$.005 < p < .01$
				13-26	$.001 < p < .005$

TABLE 5

Prenursing litter weight, lactational performance of thyro-parathyroidectomized rats during period of fatal attacks of tetany after withdrawal of orally administered A.T. 10 on day of lactation

Daily treatment for all rats T4: 3 μg/100 g. OXT: 1 U.S.P. unit	Rats	Day 16			Day 17			Day 18			Day 19		
			Milk yield			Milk yield			Milk yield			Milk yield	
		Pre-nursing litter wt.	Total	Total as % of post-nursing litter	Pre-nursing litter wt.	Total	Total as % of post-nursing litter	Pre-nursing litter wt.	Total	Total as % of post-nursing litter	Pre-nursing litter wt.	Total	Total as % of post-nursing litter
	(No.)	*(g.)*	*(g.)*	wt.	*(g.)*	*(g.)*	wt.	*(g.)*	*(g.)*	wt.	*(g.)*	*(g.)*	wt.
Intact controls	15	163 ± 4	12.5 ± .6	7.1 ± .3	170 ± 4	14.7 ± .8	8.0 ± .4	178 ± 4	16.3 ± .8	8.4 ± .4	186 ± 4	17.7 ± .7	8.7 ± .2
TPEC: Day 7, died with tetany		(34) 165 ± 4	(34) 12.9 ± .9	(34) 7.3 ± .2	(28) 165 ± 4	(28) 14.0 ± .6	(28) 7.8 ± .3	(20) 172 ± 5	(20) 14.1 ± .9	(20) 7.5 ± .3	(8) 179 ± 12	(8) 12.8 ± 1.1	(8) 6.7 ± .5

A.T. 10 = dihydrotachysterol; OXT = oxytocin; T4 = thyroxine; TPEC = thyro-parathyroidectomy; figures in parentheses = number of rats.

cessfully, had highly significant reduced blood Ca levels (Table 4), as compared to intact controls and rats with presumably accessory parathyroid tissues.

DISCUSSION

Data presented is first report of completely successful replacement therapy with A.T. 10 in lactating TPEC rats (Tables 1 and 4). Lactational performance sustained by replacement therapy was actually in excess of normally lactating rats receiving no thyroxine or oxytocin (Table 1). This was made possible by concurrent administration of optimal amounts of T4, 3 μg/100 g/day (5) and by complete evacuation of milk by young under beneficial effect of OXT, 1 U.S.P. unit/nursing time (2).

TABLE 6

Average body weight of lactating thyro-parthyroidectomized rats after withdrawal of orally administered A.T. 10 replacement therapy during period of restricted feed intake (10 hr/day)

Daily treatment for all rats T4: 3 μg/100 g. OXT: 1 U.S.P. unit	Rats	Mother's avg. body wt. (Days 16-21)			Mother's body weight		
			Evening				
		Morning	Pre-nursing	Post-nursing	Day 14	Day 21	Gain or loss
	(No.)	*(g.)*	*(g.)*	*(g.)*	*(g.)*	*(g.)*	*(%)*
Intact controls	15	266	322	302	297	301	1.6 ± 1.2[1]
TPEC: Day 7, presumably accessory parathyroid tissue	9	254	306	289	279	289	3.7 ± 1.3
TPEC: Day 7, nonfatal attacks of tetany	11	247	285	273	281	265	−5.5 ± 1.3[2]
TPEC: Day 7, no visible attacks of tetany, but presumably no accessory parathyroid tissue	10	244	281	263	269	261	−2.7 ± 1.1[3]

Student's "t" test:
1-2 $< .001$
1-3 $.01 < p < .025$

A.T. = dihydrotachysterol; OXT = oxytocin; T4 = thyroxine; TPEC = thyro-parathyroidectomy.

Although A.T. 10 is chemically a mixture of steroids related to D vitamins (6) and, therefore, very different from PTH, a polypeptide (7), and since PTEC is considered complete, this compound was as effective when given orally in amounts of 60, 80, and 100 μg/100 g/day, respectively, as subcutaneously injected parathyroid hormone, 2 × 40 U.S.P. units/day, which corresponds to 29-30 U.S.P. units/100 g/day, under identically standardized experimental conditions (9). These data indicate that about 2 μg. A.T. 10 (orally) are equal, physiologically, to 1 U.S.P. unit of parathyroid hormone (by injec-

tion) in the rat. Subcutaneous injection of A.T. 10, 187.5 μg/day, or more than 60 μg/100 g/day, gave unsatisfactory results, confirming earlier work (1).

Although 60, 80, and 100 μg. A.T. 10/100 g/day showed practically the same positive effect on lactational performance during the replacement therapy period, surviving time of the TPEC lactating rats was somewhat prolonged after administration of higher levels, confirming the well known, long-lasting effect of this compound. In contrast herewith, TPEC lactating rats, given PTH replacement therapy, died with tetany only a few hours later, when this hormone was discontinued on Day 13 when young nursed continuously (Table 3) (9). If PTH was discontinued on Day 14, survival time was markedly increased. This was ascribed tentatively to gradual inhibition of lactation and, presumably, corresponding reduction of blood calcium removal during 10-hr. separation period (9). This might be true for this experiment and explain, at least partially, long survival time of intensively lactating TPEC mothers after withdrawal of A.T. 10 replacement therapy on Day 13.

More difficult to explain is mean increase in milk yield on Day 15 as compared to Day 14 in TPEC rats, confirming earlier results (9). Differences are significant and restricted to successfully operated rats.

Post replacement therapy period and blood Ca determinations were effective in aiding in distinguishing animals with accessory parathyroid tissues and rats with low blood calcium levels due, it is believed, to complete removal of parathyroids and high losses of Ca by milk synthesis. Furthermore, data collected during this period prove that the very best lactators died with tetany, whereas rats with reduced lactational performance escaped fatal attacks.

It is difficult to explain the adverse effect on body weight of TPEC lactating rats, even when replacement therapy was successful (Table 6) (8, 9). In contrast, intact lactating control rats always gain slightly.

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TECHNICAL NOTES

OBSERVATIONS ON MILK PROTEASE [1]

Proteolytic activity in raw milk has been reported by several investigators (1-4, 8, 9). However, in some instances, it is difficult to evaluate the results of previous workers because of possible interference of bacterial protease (1-3, 9). The work of Storrs and Hull (8) appears to be a substantial contribution to the belief that protease is a natural constituent of milk. These authors found that tyrosine-tryptophane increases occurred in three milks incubated at 100° F. for 6 hr. Microbial action would not account for the changes noted, because the milks had low bacterial counts (less than 10,000 microorganisms/milliliter) and contained a mixture of bacitracin and polymyxin to inhibit bacterial growth. However, the limited data of Storrs and Hull, and the fact that their colorimetric method (which uses Folin's Phenol reagent) is lacking in specificity (7), suggested the need for this further investigation.

In one experiment, six samples of fresh milk, obtained aseptically from mastitis-free individual cows, were examined for protease activity immediately after milking, as follows: (a) equal volumes of milk and 0.2 *M* borax buffer, pH 8.5, were combined and incubated 6-8 hr. at 38° C., (b) the proteins were precipitated with 10% trichloracetic acid, (c) the filtrates thus obtained were extracted four or five times with ethyl ether, concentrated by freeze drying, resuspended in 10% isopropanol, and examined by paper chromatography techniques according to the method of McFarren (6). Proteolytic activity was expressed as μg. tyrosine/5 ml. original milk sample. Paper chromatography employing a m-cresol saturated buffered system (pH 8.4) revealed that tyrosine was the only free amino acid to increase appreciably in concentration. The increases in free tyrosine, as determined by an electrodensitometer ranged from 1.5 to 23 μg/5 ml. milk. Small but significant increases in concentrations of three peptide fractions were observed also, using a saturated buffered phenol system (pH 12.0). These peptides did not separate with the m-cresol solvent; however, Rf values of approximately 0.48, 0.37, and 0.13 were obtained with the phenol solvent. The tyrosine values agreed with those found by the Hull method (5).

To obtain further evidence as to the presence of protease in milk, additional samples were analyzed by the stated procedure, with the exception that the colorimetric method of Hull (5) was substituted for paper chromatography. Also, raw milk treated with 1% chloroform to inhibit microbial growth and milk pasteurized at 145° F. for 30 min. were incubated and analyzed. The results for protease activity and initial bacterial counts are in Table 1. The total plate counts were not more than 16,000/milliliter at the beginning of analyses and less than 300/milliliter after incubation; whereas, the proteolytic plate counts were 4,000/milliliter or less initially and less than 300/milliliter after incubation. These decreases were attributed to the action of the borax buffer which was strongly inhibitory to the microflora of milk. There was no relationship between proteolytic activity and the initial bacterial numbers. For example, the raw milk sample with 3,500 proteolytic bacteria/milliliter had a proteolytic activity of 12 μg/5 ml., whereas the sample with the 126 μg/5 ml. protease activity had a proteolytic count of less than 300/milliliter. For the raw milk samples, the proteolytic activity expressed as free tyrosine ranged from 4 to 126 μg/5 ml., with an average of 57.0 μg/5 ml. Neither the addition of chloroform nor pasteurization had

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TABLE 1

Proteolysis and bacteriological flora in raw, chloroform-treated raw, and pasteurized milk

Sample	No. of samples	Proteolytic activity [a] μg. tyrosine/5 ml.		Initial bacterial counts [b]			
				Total bacteria		Proteolytic bacteria	
		Range [c]	Average	Range	Log. av.	Range	Log. av.
Whole raw milk	47	4-126	57.0	<300-16,000	3,500	<300-3,500	750
Raw milk + 1% chloroform	25	6-124	55.2	<300-12,500	3,900	<300-4,000	1,100
Pasteurized milk	20	8-120	51.8	<300-3,700	780	<300-400	225

[a] Standard deviation for method ± 4 μg/5 ml.

[b] After incubation all counts were less than 300/milliliter.

[c] Six samples less than 10 μg/5 ml. tyrosine for raw milk; three samples less than 10 μg/5 ml. tyrosine for raw milk + chloroform; three samples less than 10 μg/5 ml. tyrosine for pasteurized milk.

any pronounced effect on proteolytic activity. Thus, the enzyme appears to be resistant to heat, although it was essentially inactivated by 194° F. for 5 min.

Generally, the results reveal that raw millk usually (but not always) contains a small and variable amount of protease enzyme. The proteolysis is so slight as to indicate that this enzyme does not appear to have a significant role in major changes in the milk system or in dairy products. However, one must keep in mind that the magnitude of a substance in a system is merely an expression of the sensitivity of the method used. Therefore, more sensitive methods and different conditions may reveal, as yet, unknown facts about this apparent milk component.

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OBSERVATIONS ON THE ABSORPTION OF GLUCOSE INTO THE PORTAL VEIN[1]

Studies in sheep have shown that arterio-portal glucose differences are practically nil, indicating that glucose is of little importance as an end product of carbohydrate digestion (1, 8). However, limited data presented in this paper indicate that it may be of importance under some conditions.

A 500-lb. Holstein calf in which a portal vein cannula and an exteriorized carotid artery had been established (3) was used. The portal cannula was established with the tip resting in the porta hepatis. However, at autopsy, the tip was located immediately posterior to the gastrosplenic vein and conclusive evidence has been obtained indicating that it was in this position when these studies were conducted (3). Therefore, the portal data represent only the blood draining the intestines.

Two experiments were conducted using each of two rations—2.5 lb. corn and 8.0 lb. alfalfa hay or 2.5 lb. corn and 8.0 lb. timothy hay. Half of the ration was fed twice daily. Portal and carotid blood samples for glucose analysis (6) were obtained at 3-hr. intervals from 0 to 12 hr. after feeding. At least a ten-day period was allowed before the first experiment with each ration.

With one exception, the portal-carotid glucose difference was positive when alfalfa hay was fed (Table 1). The average difference of 5.4 mg. % was statistically significant ($P < 0.05$). These differences were smaller and often negative when timothy hay was fed and the −1.7 mg. % average difference was not significant. The portal-carotid difference was greatest in the earlier samples and decreased with increased time after feeding. Most of this decrease was associated with a rise in the carotid glucose level, while the portal glucose level remained fairly stable. Average portal glucose levels were similar in all experiments. The carotid glucose levels were considerably higher when timothy hay was fed, which accounted for the lower average portal-carotid difference with this ration.

While no blood flow figures are available for the portion of the portal system sampled in this study, a rough estimate may be made from

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TABLE 1
Average portal vein and carotid artery glucose levels and portal-carotid glucose differences at various times after feeding

	Hours after feeding					
	0	3	6	9	12	Average
			Alfalfa hay and corn[a]			
Portal (mg. %)	74.6	76.3	74.5	79.3	75.7	76.3
Carotid (mg. %)	68.0	68.4	67.3	75.2	77.4	70.9
Portal-carotid (mg. %)	6.6	7.9	7.2	4.1	−1.7	5.4
			Timothy hay and corn[a]			
Portal (mg. %)	77.8	76.6	74.1	79.2	71.7	76.2
Carotid (mg. %)	74.0	77.8	76.6	81.2	78.1	77.9
Portal-carotid (mg. %)	3.8	−1.2	−2.5	−2.0	−6.4	−1.7

[a] Average of two experiments.

the difference between the total portal flow (5) and the gastrosplenic flow (4). From this it was estimated that approximately 300 g. of glucose per day were absorbed when the alfalfa-corn ration was fed. Assuming that glucose is equivalent to 3.8 cal/g and TDN to 1,814 cal/lb (2), the glucose would account for approximately 10% of the energy available to the animal. McGilliard (7), in studies with a duodenal fistula animal fed a ration of 70% alfalfa and 30% corn, found that 19% of the NFE of the ration disappeared in the lower tract. If a similar proportion of the NFE disappeared from the lower tract on our studies, it would be more than sufficient to provide the 300 g. of glucose.

If the origin of the glucose in the alfalfa-corn ration was the starch of corn, the failure to obtain significant glucose absorption with the timothy corn ration would imply that the type of roughage influences the passage of corn from the rumen. This would be of considerable practical importance because starch utilized as glucose would provide more energy to the animal than starch which had been fermented to volatile acids in the rumen.

No consideration has been given to possible hemodilution in the portal vein (5). If it occurs, it would increase the estimates of glucose absorption.

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TECHNIQUE FOR OBTAINING SERIAL SAMPLES OF ARTERIAL BLOOD FROM THE MIDDLE COCCYGEAL ARTERY IN THE BOVINE[1]

Studies dealing with the physiological variations in the relative amounts of various blood constituents in arterial and venous blood have brought about the need for a convenient technique for obtaining serial samples of arterial blood.

Blackwood and Stirling (1) took arterial

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blood from the radial artery of lactating and nonlactating cows, but found arterial puncture of nonanesthetized cows to be rather difficult. Lintzel (5) reported that he obtained arterial blood from goats and cattle by puncture of the heart chamber. Graham *et al.* (3) obtained arterial blood from the internal iliac artery, but had to puncture the rectal wall, with the resulting possibility of infection into the blood stream. Graham *et al.* (4) described an operation by which the carotid artery was exteriorized and sewed into a skin flap which gave a convenient place to obtain arterial blood. Boyd and Petersen (2) reported a technique for puncture of the external pudic artery. Saarinen (7) described a simple method for obtaining arterial blood from the cow's tail.

This paper reports further work using the method first described by Saarinen for obtaining arterial blood. Studies being conducted called for samples of arterial blood at 2-hr. intervals over 24-hr. periods. Using the method described by Saarinen, it was possible to obtain arterial blood every 2 hr. for only about 6 hr., but after this time small hematomas formed. The next procedure was catheterization. The techniques used in venous catheterization in several species have been reviewed by Ralston *et al.* (6) in connection with their work in catheterizing the jugular veins of dairy cows.

The procedure used in this study was to clip the cow's tail and clean the ventral surface with 70% ethyl alcohol. The ventral area was then anesthetized by local procaine infiltration (2 ml. of a 1% solution). The location of the middle coccygeal artery which runs through the ventral (hemal) arches of the coccygeal vertebrae was then determined by palpation with the fingertips on the ventral surface of the fourth to seventh coccygeal vertebra. After the artery was located, a sterile 16-gauge thin-wall needle 2 in. in length, with a short beveled point, was introduced into the artery at approximately a 30° angle in a craniad direction, with the short edge of the bevel placed in the direction in which the catheter was to travel. Entrance into the artery was evidenced by a strong spurting blood flow. It was necessary to exert care to keep the needle in the artery when the animal moved. At this point, sterile polyethylene tubing (PE-90/S12 of 12 in. length) was threaded through the needle toward the heart with the beveled end in the same direction as that of the needle. The warm blood flowing through the tubing made it very soft and flexible and it was often difficult to thread through the needle unless a 23-gauge piece of sterile stainless steel wire was inserted through the tubing. The wire was removed as 7 to 8 in. of the tubing was threaded into the artery. The 16-gauge needle was then removed from the artery. A blunt-nosed 20-gauge needle was then placed in the end of the tubing, which made the injection of small amounts of a diluted heparin solution (100 U.S.P. units per 100 ml. of 0.9% saline) into the tubing and the removal of blood very convenient. After withdrawal of approximately 25 ml. of blood into a 30-ml. heparinized syringe, the tubing was filled with a diluted heparin solution to wash out the blood and prevent the formation of a clot. This procedure was followed at each subsequent sampling. The tubing was then folded near the end of the 20-gauge needle and clamped off with a bull-dog clamp. The tubing, needle, and clamp were then taped to the tail with adhesive tape to prevent fecal contamination and the possible removal of the tubing until the next desired sampling time.

No difficulties were encountered when the tubing remained in the artery over 24-hr. periods.

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DEVELOPMENT OF ZINC DEFICIENCY IN HOLSTEIN CALVES FED A PURIFIED DIET

Zinc has been shown to be a required nutrient for many species and is believed to be essential for all animals. As late as 1956, it was stated by leading authorities that a deficiency of this element in farm animals was unlikely under practical conditions (3, 7). Since then it has been found that zinc deficiency often occurs under practical conditions in swine (6). The authors have been unable to find a report of a zinc deficiency in cattle or any other ruminant animal. An experimentally produced zinc deficiency in male Holstein calves is described in this report.

The four experimental calves stayed with their dams for one day after birth and were then given a total of 500 lb. whole milk each over an 8-wk. period. They were maintained in wooden pens with elevated slatted bottoms without bedding. Milk from cows in the middle portion of lactation was used, as it has been reported that the zinc content of milk is at its lowest during this period (5). Care was taken to prevent contamination of the milk with zinc from the milking equipment and plastic buckets were used to feed both the milk and water. Stream water obtained from the city supply through a system containing no galvanized pipe was used.

Beginning the fifth week of the trial and continuing for 12 additional weeks, two of the calves were given a low zinc purified diet, while the other two received the same diet supplemented with 2.3 g. ZnO per 100 lb. feed. The purified diet, based on those of Edwards *et al.* (1) and Matrone *et al.* (2), was composed of the following: egg albumin (autoclaved), 16.0 lb.; glucose monohydrate, 60.0 lb.; lard, 3.0 lb.; cellulose,[1] 5.0 lb.; $KHCO_3$, 4.5 lb.; $NaHCO_3$, 7.5 lb.; $CaHPO_4$, 844 g.; KCl, 282 g.; NaCl, 247 g.; $MgSO_4 \cdot nH_2O$, 210 g.; $FeSO_4 \cdot nH_2O$, 7.89 g.; $MnSO_4 \cdot H_2O$, 1.44 g.; $CuSO_4 \cdot 5H_2O$, 920 mg.; $CoCl_2 \cdot 6H_2O$, 9 mg.; KI, 8 mg.; thiamine HCl, 410 mg.; riboflavin, 880 mg.; niacin (80%), 1.17 g.; Ca pantothenate, 1.47 g.; choline chloride, 119.0 g.; pyridoxine HCl, 590 mg.; folic acid, 60 mg.; biotin, 12 mg.; menadione, 120 mg.; 0.1% B_{12} with dicalcium phosphate, 4.88 g.; α-tocopheryl acetate (276 U/g), 600 mg.; vitamin A (300,000 U/g), 800 mg.; vitamin D-3 (200,000 U/g), 800 mg.; Terramycin HCl (895 U/mg.), 5.00 g.; and Oleandomycin (25%), 2.00 g.

Milk, water, blood, and feed were analyzed for zinc by the dithizone method of Verdier *et al.* (8) and blood was analyzed for carbonic anhydrase activity by the method of Pincus *et al.* (4). The average zinc contents of the milk, basal diet, basal diet with zinc added, and the water were 3.9, 2.7, 46.0, and < 0.1 p.p.m., respectively.

[1] Solka-floc, manufactured by the Brown Company, Berlin, New Hampshire.

RESULTS AND DISCUSSION

The control calves made satisfactory growth on the purified diet. During this period those receiving the low-zinc ration did not gain as rapidly as the controls (Table 1). The calves obtained 0.8 to 0.9 g. zinc from the milk, which was about two-thirds of the total received by those fed the low-zinc diet.

When milk feeding was discontinued, the hair of all calves developed a rough condition which disappeared after a few weeks. Except for excessive salivation, no abnormalities in the gross appearance were observed until the calves had received the purified diet for 8.5 wk. About this time the following symptoms were observed in those fed the low-zinc diet: slightly red and inflamed nose and mouth; soft swelling above the rear feet in front of the fetlock; small areas of alopecia on the rear legs; breaks in the skin around the hoofs; rough, scaly, skin on the rear legs; and dull and listless appearance. The skin on the neck of Calf No. 3 soon became very rough and lumpy, so that collection of blood samples from this animal was difficult, as bits of flesh usually plugged the needle.

A complete examination of all calves was made by personnel of the School of Veterinary Medicine after the purified diets had been fed

TABLE 1
Zinc intakes and body weight gains

	1 Control	2 Control	3 Low Zn	4 Low Zn
Age at end of trial *(wk.)*	19	16	18.5	18
Zn intake from water *(g.)*	0.09	0.15	0.08	0.16
Zn intake from milk *(g.)*	0.91	0.80	0.91	0.86
Zn intake from feed *(g.)*	6.28	4.23	0.34	0.32
Total Zn intake *(g.)*	7.28	5.18	1.33	1.34
Body weight gain *(lb/wk)*	10.0	10.9	7.7	8.4

for 11 wk. The following symptoms were observed for the calves fed the low-zinc diet: stiff gait; swelling of the hocks and knees; soft swelling above the rear feet in front of the fetlocks with an accumulation of fluid; creased, wrinkled appearance of skin on the legs with red coloration where hair had come out in patches (these patches were larger and more abundant on the rear legs); red, scabby, and shrunken skin on the scrotum (Figure 1); hemorrhages around the teeth; and ulcers on the dental pad. The symptoms developed by Calf No. 3 were more severe than those exhibited by Calf No. 4. In addition, this individual exhibited dilation of the pupils, apparent impairment of vision, rough and thickened skin, and dry, lifeless hair (Figure 2). On gross examination, the zinc-deficient calves exhibited many of the symptoms that have been reported for hyperkeratosis. The two calves receiving the ration with added ZnO were normal in appearance except that the inside of the mouth and the gums were slightly pink.

At the end of 12 wk. on the purified diet all the calves were slaughtered for post mortem examination and histopathological studies. The skin of the zinc-deficient calves showed evidence of parakeratosis, with a reduced number of cells in the stratum granulosum. There was acanthosis and elongation of the rete net with excessive keratin formation and retention of nuclei, with the appearance of some edema in these tissues. The papillae of the rumen showed excessive over-growth, a moderate keratin formation, and retention of nuclei. Although all of the calves exhibited some parakeratosis of rumen walls and of the esophageal mucosa, the two controls appeared normal in other respects. It is probable that the conditions of the rumens were due to the lack of roughage in the diet.

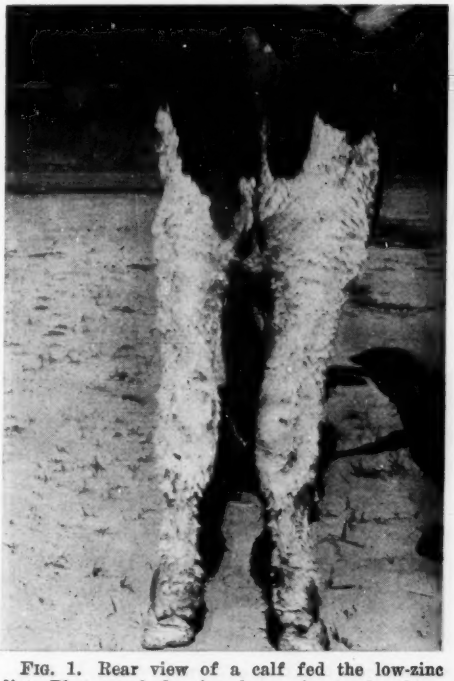

FIG. 1. Rear view of a calf fed the low-zinc diet. Photographed a few days prior to slaughter.

FIG. 2. Head view of a calf fed the low-zinc diet. Photographed a few days prior to slaughter.

The zinc content and carbonic anhydrase activity of the blood from the calves fed the low-zinc diet were statistically significantly lower than those of the controls. For the last 2 mo. of the trial the average values of blood zinc in μg/ml and carbonic anhydrase activity in EU/ml (Enzyme Units) were 2.9, 482, 1.7, and 398 for the control and low-zinc calves, respectively. Corresponding values for the previous 4 wk., when they had been receiving the diets for only a short time, were 3.0, 469, 2.5, and 495.

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INTERPRETIVE SUMMARIES OF PAPERS

BINDING OF RIBOFLAVIN AND RIBOFLAVIN PHOSPHATE BY THE PROTEINS OF MILK

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Interaction between proteins and small organic molecules is capable of modifying profoundly the properties of proteins. The phenomenon lends itself to practical application in the stabilization of serum albumin in solution against heat. In dairy technology the problem of stabilizing milk proteins against coagulation differs from that of stabilizing serum albumin. The proteins in milk are present largely in the form of so-called micelles, and it is the stabilization of these micelles against aggregation simultaneously with the preservation of the integrity of the individual micelles which poses the problem. Some workers believe that the micelles in milk owe whatever stability they possess to an ordered structure such as one finds in soap micelles. This raises the question whether the protein micelles possess the extraordinary binding and solubilizing characteristics usually found in organized micellar structures. Studies on the binding of riboflavin and riboflavin phosphate by the proteins of milk failed to support the hypothesis of an ordered micellar structure. The binding by the calcium caseinate–phosphate complex in milk was found to be equivalent to the binding by its component protein molecules.

Although the binding by milk proteins is of a low order of magnitude, the quantity of riboflavin bound at low temperatures comprises 25-30% of the total riboflavin and presumably this amount would be reflected in the partition of riboflavin in curds and whey in the manufacture of cheese and casein.

The degree with which both riboflavin and riboflavin phosphate are bound decreases rapidly as temperature is increased. Thus, at sterilizing temperatures binding is negligibly small. Because of the high negative temperature coefficient, it is doubtful that binding by proteins is of importance at sterilizing temperature and, therefore, the stabilization of the protein complex in milk against heat coagulation by riboflavin phosphate (and presumably by other biologically important organic phosphates) must reside as it does in the instance of inorganic phosphate in the ability of these compounds to bind calcium.

The photochemical decomposition of riboflavin is inhibited by the caseins in a manner indicating that the degree of inhibition is related to the degree of binding.

ACTION OF RENNIN AND PEPSIN ON β-CASEIN: INSOLUBLE AND SOLUBLE PRODUCTS

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When skimmilk is coagulated with rennin or pepsin, *beta*-casein is included in the clot. *Beta*-casein alone, with the same concentration of calcium ions, however, is not coagulated by these enzymes. Researchers studying this reaction

have concluded that rennin and pepsin do not act on *beta*-casein since soluble split products were not found. The enzymatic coagulation of *alpha*-casein is accompanied by the appearance of a soluble fraction. The present research shows that rennin and pepsin do act on *beta*-casein and when 2% trichloroacetic acid or adjustment to pH 4.7 are used as precipitants, the appearance of a soluble fraction is evident. Thus, *beta*-casein, when coprecipitated with the total casein in the clotting of skimmilk, has undergone some alteration from its native state. This change must be considered in evaluating the properties of the enzymatic clot of milk.

NITROGEN FRACTIONS IN CENTRIFUGE PREPARATIONS FROM MILK

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A study was made to ascertain some effects of certain types of centrifugal action on the subsequent distribution of protein fractions within the centrifuged milks. Effects of room or refrigerator temperatures, both while centrifuging and also during storage before centrifuging, were investigated. The centrifugal actions used were those of a commercial cream separator, a Sharples supercentrifuge, and a Ford air-driven bowl. Both the Sharples and the Ford instruments were used as batch type separators by first filling the slowly rotating bowl and then bringing it up to speed. The Sharples instrument was also used with continuous flow through the rapidly rotating bowl. It was used both at about 27° C., and when cooled with circulating ice water.

The nitrogen fractions determined by the method of Aschaffenburg and Drewry were casein, noncasein, nonprotein, β-lactoglobulin, globulin, residual albumin, and proteose-peptone. Their distribution, both in absolute amounts and as ratios, differed among the preparations.

IDENTIFICATION OF SOME VOLATILE CARBONYL COMPOUNDS FROM NONFAT DRY MILK

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The recent development of economical instant forms of nonfat dry milk (NFDM) constitutes an important technological advance in the dairy industry. Instant milk has much more household utility than conventional spray dried milk and there is an apparent growth in the use of this product for reconstituted beverages. This trend places a high premium on flavor quality. There is general recognition that instant NFDM has distinctive flavor. The flavor has been described by such terms as hay-like, cereal, malty, and stale. The flavor intensity varies among brands and the terminology is apparently influenced by the intensity. Except in cases of very intense flavor, this characteristic probably should not be considered a defect. The distinctive flavor of reconstituted instant NFDM would be considered a defect only if it has a

significant retarding effect on the acceptance of reconstituted NFDM as a substitute for fresh fluid skimmilk.

The first successful instant NFDM's were prepared by processes which involved the wetting of conventional spray dried milk with wet steam to added moisture levels of 5 to 15%. The resulting agglomerated powder particles were then redried in hot air to yield large particles of relatively low density and instant dispersibility in cold water. Later processes have tended toward continuous operation by modifying the original spray drying operation to yield large agglomerated particles by such techniques as the use of low-pressure nozzles and recirculation of fines into the wet milk spray. A common feature of all of the processes is that they involve relatively slow drying during the final stage of high concentration of milk solids. This naturally accentuates the possibility of heat-induced chemical reactions during this critical stage.

The objective of the present study was to identify some of the volatile flavor compounds from NFDM, with the expectation that this would help to define the chemical mechanisms leading to flavor production. Better understanding of these mechanisms would provide basic knowledge to be applied in possible manufacturing and packaging developments in future dry milk technology.

The results of this study have indicated that volatile carbonyl compounds are significant contributors to the characteristic flavors of instant NFDM. These compounds apparently arise from at least two different chemical phenomena. Some of them result from Maillard-type browning reactions involving lactose and milk protein interactions. Other identified compounds appear to result from lipid oxidation reactions. Evidence of the involvement of the browning reaction in flavor production confirms previous indications obtained by independent investigation. Practical efforts to minimize browning manifestations in instant milk have already been initiated in the industry by processes which accelerate the instantizing and which hold heat treatment to a practical minimum. The discovery that lipid oxidation is probably involved in flavor production is worthy of special consideration. The current practice of air-packing might indicate that the industry does not recognize the possibility of significant flavor production from lipid oxidation. It is recognized that vacuum or inert gas packing is expensive and probably should not be commercially considered at the present time. However, it is suggested that research on methods to minimize lipid oxidation in NFDM is definitely worth while. It may be possible to produce NFDM which is lower in fat and phospholipid content than present commercial products (0.3 to 0.5%). Such a step might remove a significant amount of flavor precursor.

FATTY ACID COMPOSITION OF THE MONOGLYCERIDES FROM LIPOLYZED MILK FAT

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Although monoglycerides are produced along with free fatty acids and diglycerides when milk fat is digested by milk lipase, the fatty acid composi-

tion of these compounds has not been reported to date. This paper reports a study wherein the fatty acids were identified.

Monoglycerides, which consist of a single fatty acid attached to glycerol, were concentrated from rancid milk fat and converted to allyl esters, a form which lends itself to analysis by gas-liquid chromatography. Nineteen of 27 esters found were identified. Whereas all of the fatty acids found are known to occur in milk fat, the patterns were distinctly different. Palmitate, oleate, and myristate were present in the largest quantities. All of these compounds are surface-active. Some of the short-chain monoglycerides found may contribute to the flavor of rancid milk. The procedure should be useful in studies of milk lipase specificity and milk fat molecule structure.

SPECIFICITY OF MILK LIPASE TOWARD THE PRIMARY ESTER GROUPS OF SOME SYNTHETIC TRIGLYCERIDES

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Almost nothing is known about the sequence by which the fat-splitting enzyme, milk lipase, hydrolyzes or digests fat molecules. This paper describes a study in which two synthetic fats were digested by milk lipase in order to determine the sequence of digestion.

Two fats, one with oleic acid in the center position on the glycerol portion of the molecule and palmitic acid in the two outside positions, and one with the opposite configuration, were digested by milk lipase. The products of digestion were recovered and analyzed for fatty acid composition with gas-liquid chromatography. The results indicated that with the two fats studied, milk lipase preferentially hydrolyzed fatty acids from the two outside positions of the glycerol portion of the fat molecules. If the results are applicable to milk fat, then positional specificity may partially explain some of the differences noted between samples of rancid milk containing the same quantities of free fatty acids.

INFLUENCE OF ALFALFA AND OAT HAYS ON SUSCEPTIBILITY OF MILK TO OXIDIZED FLAVOR

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Milk produced under the dry-lot feeding system commonly used in California's market milk dairies is highly susceptible to oxidized flavor. This off-flavor seriously limits the time that can be allowed between production at the farm and processing in the dairy plant, and retards adoption of economies such as alternate-day milk collection and less frequent processing in the dairy plant. Before recent improvements in refrigeration and sanitary methods of milk production, bacteria commonly limited usable life of milk, but flavor defects

that develop during storage as a result of chemical changes are now frequently of greater concern to the dairy producer.

Feeds are known to influence susceptibility of milk to oxidized flavor. In general, green succulent feeds yield milk with greater resistance to the defect than dry feeds. Even in comparison of dry roughages, there appear to be appreciable differences in susceptibility. Unfortunately, reliable information is not available to guide producers in selecting feeds that produce milk with greater resistance to oxidized flavor. The principal objectives of this study were to compare oat hay with alfalfa hay in relation to oxidized flavor and, as a guide in selecting feeds, to identify the chemical constituents in the feeds that are associated with high resistance to the defect.

In an 18-wk. feeding trial, milk produced on oat hay was much more resistant to oxidized flavor than that produced on alfalfa hay. Significant differences related to the ration were found in the natural copper, carotene, and tocopherol contents of the milk, and in the oxidative stability and unsaturated fatty acid composition of the milk fat. However, additional work is needed to determine the relative importance of the various constituents in determining susceptibility of milk to oxidized flavor.

HEAT RESISTANCE OF THREE STRAINS OF PSYCHROPHILIC ORGANISMS ADDED TO SKIMMILK FOR COTTAGE CHEESE MANUFACTURE

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Spoilage that occurs in Cottage Cheese under present marketing conditions is primarily the result of growth of psychrophiles. Microorganisms of the genus *Pseudomonas*, yeasts, and molds are representative. Thermal resistance studies of *Pseudomonas viscosa*, *Pseudomonas fluorescens*, and *Pseudomonas fragi*, known spoilage organisms of Cottage Cheese, were made in sterile skimmilk at 110, 115, and 120° F. In addition, the resistance to heat of these organisms during the process of making Cottage Cheese was also determined by inoculating the fresh pasteurized skimmilk with a culture of each organism at the time of setting. The level of inoculation of *Pseudomonas* was considerably above that expected from normal contamination of pasteurized milk.

The heat resistance of the three test organisms in sterile skimmilk are characterized by the D and z values calculated from survival curve data. These results provide evidence that regular pasteurization temperatures used for skimmilk for Cottage Cheese would destroy these organisms. In the presence of acid, of concentrations common to Cottage Cheese manufacture, the heat resistance of these organisms is markedly reduced. None of the species survived the cooking process of 120° F. for 30 min. In fact, at time of cutting the curd at pH 4.7 with no change in temperature, there had been a 90% reduction in *Pseudomonas* count as compared with that at time of setting. As further

evidence of their lack of survival, none of the organisms added to the fresh skimmilk could be recovered from lots of creamed curd stored at 38° F. for 4 wk. and tested at weekly intervals.

AVAILABILITY AND METABOLISM OF VARIOUS SUBSTRATES IN RUMINANTS. III. THE EFFECT OF PROPIONATE ON ACETATE OXIDATION IN VIVO

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Acetic acid and propionic acid are absorbed from the rumen into the blood where they are available as primary substrates for animal tissues. In vitro studies indicate that acetate oxidation is markedly depressed in the presence of propionate. The studies reported in this paper were undertaken to determine if propionate has a similar effect on acetate oxidation in the intact animal.

The results indicate that acetate oxidation is depressed when propionic acid is infused along with acetate. However, this does not appear to be a specific effect on acetate oxidation, but rather a reduction in the over-all metabolism, as indicated by the lower total carbon dioxide production when propionate was infused with acetate. The data show that the percentage of carbon dioxide arising from the oxidation of acetate carbon was the same when acetate was infused alone or with propionate. Thus, acetate contributed proportionately the same amount to the expired carbon dioxide during both treatments, even though the total carbon dioxide production was less when propionate was infused. This is clearly demonstrated by the marked similarity of the relative specific activity curves of the expired CO_2.

The increased carbon dioxide excretion when acetate was infused alone is in agreement with the known high specific dynamic effect of acetate. The modifying effect of propionate is in agreement with the reported fact that the efficiency of acetate utilization is markedly increased in the presence of propionate.

PRODUCTION AND ABSORPTION OF VOLATILE FATTY ACIDS IN THE PERFUSED RUMEN

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The short-chain volatile fatty acids, chiefly acetic, propionic, and bu'yric, are recognized as the principal digestion products of dietary carbohydrate in ruminants. Each acid is capable of entering into a series of chemical reactions through which useful energy or substrate is furnished to the animal. However, there are differences in the reaction sequences and, thus, the efficiency with which acetic, propionic, and butyric acids are utilized. The value of a ration

for ruminants then depends in part on the total and relative amounts of the short-chain volatile acids that become available to the animal as a result of carbohydrate digestion in the rumen.

Volatile acid production is not easily measured in the living animal because the acids are being utilized as rapidly as they are produced. Perfusion of the isolated rumen permits continued production in the absence of acid-utilizing systems. The perfusion technique also makes possible a study of the rates at which the volatile acids are absorbed into the blood, e.g., the rates at which they are made available to the animal.

The perfusion studies reported here show that the volatile acids are produced in the same relative amounts as they occur in rumen fluid and that their absorption into the blood is proportional to their concentration in the rumen. When the concentration of either propionic or butyric acid in rumen fluid was greatly increased by adding the appropriate acid to the rumen, a corresponding increase in the added acid was measured in the rumen vein blood. Butyric acid is of particular interest in this respect, since some other investigators have reported low levels of butyric acid in rumen vein blood and attributed these low values to partial oxidation of butyric acid to β-hydroxybutyric acid by rumen epithelial tissue. However, the perfusion studies reported here are in excellent agreement with the perfusion studies reported by the Maryland group.

EFFECT OF PHLORIDZIN, INSULIN, AND BUTYRATE ON THE CONCENTRATION OF GLUCOSE AND KETONES IN THE BLOOD AND URINE OF FASTED STEERS

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The specific factor or factors responsible for the development of ketosis in ruminants have not been clearly established. The experiments reported in this paper were designed to test the hypothesis that ketosis is caused by a specific demand for glucose that exceeds the capacity of the animal's tissues to mobilize this metabolite. Although other metabolites may replace glucose in many of the reactions in which it participates, there are several reactions which require glucose as an obligatory substrate. The synthesis of lactose is a reaction sequence for which glucose is apparently required; thus, the glucose demand in high-producing cows would be greatest in early lactation, when the lactation drive is at its peak. Bovine ketosis invariably occurs during the first few weeks of lactation.

Ketosis does not normally occur in steers, a fact that can be explained by the suggested hypothesis that in the growing or fattening steer there is no unusually high glucose demand. However, there is no reason to believe that ketosis would not result if a glucose drain of sufficient magnitude were imposed on a steer.

The experimental approach used in the studies reported here utilized the known physiological action of phloridzin to create a glucose drain in the steer.

Phloridzin lowers the renal threshold for glucose when administered to animals; that is, it causes glucose to be excreted in the urine even though the concentration of glucose in blood is normal or below normal. Prior to each experiment the steer was fasted 48 to 72 hr. to deplete the carbohydrate reserves. In these experiments butyrate was administered to supply a readily available ketogenic substrate.

The magnitude of the glucose stress achieved by the various treatments was indicated by the amount of glucose excreted in the urine during the treatment period. The infusion of phloridzin resulted in the excretion of 55.81 g. of glucose in an 8-hr. period. This rate of glucose excretion is comparable to the glucose demand for lactose synthesis of a 1,200-lb. cow producing 28.8 lb. of milk per day. The imposed glucose stress was mild and did not induce clinical manifestations of ketosis in the steer. However, the blood sugar concentration was depressed and there was a slight rise in blood ketones. When insulin was administered in addition to phloridzin, the blood sugar concentration decreased to levels typical of severe ketosis, but the ketone concentration in blood was less than when phloridzin was administered alone. This suggests that insulin augmented glucose utilization and depressed ketone formation.

Three experiments were conducted in which neutralized butyric acid was infused to provide a readily available ketogenic substrate. A surprising result of the butyrate infusions was the marked depression in blood sugar. In each instance when butyrate was infused, the blood sugar concentration fell rapidly to ketotic levels; whereas, the ketone concentration rose to the ketotic level only when phloridzin was administered simultaneously.

The results of these experiments show that a simple glucose stress of the magnitude induced in these trials is not sufficient to cause the clinical symptoms of bovine ketosis. The trends observed in blood glucose and ketones indicate that a more severe glucose stress may induce ketosis; however, some other trigger mechanism may be involved.

EFFECT OF DURATION OF DEFICIENT CAROTENE INTAKE UPON SUBSEQUENT UTILIZATION OF CAROTENE FROM ALFALFA BY HOLSTEIN CALVES

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Cattle are subjected at times to periods of suboptimum or deficient carotene intake which, if continued, result in vitamin A deficiency. While lowered carotene utilization has been demonstrated with cattle fed deficient carotene

intakes for extended periods of time or fed essentially carotene-free rations until practically devoid of vitamin A stores, the effect of various durations of deficient carotene intake have not been explored.

Thirty-two Holstein male calves, partially depleted of their vitamin A stores, were fed one of two initial carotene intakes, one such that a definite vitamin A deficiency would develop and one such that no deficiency would develop. Calves in each of these groups were further subdivided such that one-half received their respective carotene intake for a short period of 4 wk. and the other for an extended period of 16 wk. Thereafter, calves in each of the subgroups were fed one of two levels of carotene intake of sufficient magnitude to elicit appreciable plasma and liver vitamin A responses. This latter period was for the purpose of quantitatively measuring the ability of the calf to convert carotene to vitamin A.

Based upon blood plasma and liver vitamin A concentrations during the latter period, it was found that calves fed for 16 wk. the deficient initial carotene intake utilized carotene 60 to 80% as effectively as calves fed the adequate initial carotene intake for the same period. In possible contrast, little difference existed in the utilization of carotene between calves fed the deficient initial carotene intake for 4 wk. and those fed the adequate intake for the same period of time. Thus, it would appear that cattle can withstand relatively short periods of deficient carotene intake, but that extended periods apparently decrease their ability to utilize carotene.

A COMPARISON OF FEEDING GROUND CORNCOBS AND A LIQUID UREA-MOLASSES-MINERAL MIX WITH AND WITHOUT ETHYL ALCOHOL TO YEARLING DAIRY HEIFERS

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Ever since the last century, when it was first discovered that ruminant animals can utilize a nonprotein nitrogen source (such as urea) as a substitute for plant protein, researchers and feeders have been curious as to how and the extent to which this is accomplished.

Ten to 20 yr. ago it was accepted that urea on a crude protein equivalent basis could be satisfactorily substituted for up to one-third of the protein in the ruminant ration.

This paper indicates that urea under certain controlled conditions can provide 100% of the nitrogen or protein requirement in the ruminant ration. It also indicates that much work remains before the conditions under which urea as the sole source of retained nitrogen can be fed for optimum animal performance.

There has been some indication that ethyl alcohol in the ruminant ration might provide a more highly reduced medium in the rumen and allow fermentation to proceed more rapidly. Data were not obtained to substantiate this statement.

INFLUENCE OF PRESERVATIVES ON THE FERMENTATION, NUTRIENT RECOVERY, AND FEEDING VALUE OF ALFALFA, STARR MILLET, AND COWPEA AND SUDAN GRASS SILAGES

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The use of forages as silage remains a controversial subject due to the variable results obtained by dairymen in their use. Since direct-cut forage reduces the time and labor required in making silage, farmers prefer this system to wilting. Unfortunately, most of the difficulties in silage intake and excess loss in the silo have been with direct-cut forage. This study, which included 12 silos of silage, was initiated to study these problems. Included in the study were silages made from alfalfa, Starr millet, and a mixture of cowpeas and Sudan grass and ground snap corn, citrus pulp, molasses, and sodium metabisulfite as preservatives.

When first-cutting alfalfa was preserved with 200 lb. snap corn, 200 lb. citrus pulp, or 80 lb. molasses per ton, the respective average losses or dry matter in the silo were 7.1, 16.2, and 15.6%. These losses corresponded to the rate of increase in lactic acid in the silos during the first few days after ensiling. The millet and Sudan grass silages presented no special problems in either silage preservation or losses in the silo.

The silages were fed to dairy cows on a free-choice intake basis with grain fed according to production as the only additional ingredient in the ration. The average dry matter intake from silage was 2.37 lb. per 100 lb. body weight and indicates that direct-cut forage can be preserved without excessive loss in the silo and result in high levels of intake by use of good preservatives.

SUCCESSFUL DIHYDROTACHYSTEROL (A.T. 10) REPLACEMENT THERAPY IN LACTATING THYRO-PARATHYROIDECTOMIZED RATS

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The important role of the hormone of the thyroid gland, thyroxine, in lactation has been recognized for many years. The role of the parathyroid gland in this process is less well known. When the thyroid glands of rats are removed, the parathyroid glands also are removed. In lactating rats, the removal of these two glands results in rapid depression of litter growth due to depressed milk secretion. No one, heretofore, has been able to maintain normal lactation in thyro-parathyroidectomized rats.

The determination of optimal levels of thyroxine required for lactation of about 3-3.5 μg/100 g. body weight in the strain of rats used made possible the study of the parathyroid hormone requirements for lactation in the double-operated rats. In a previous report, it was shown that 29-30 U.S.P. units of parathyroid hormone/100 g/day with thyroxine permitted normal litter growth and lactation.

In the present study, it was shown that an irradiated plant sterol called dihydrotachysterol (A.T. 10 or Hytakerol) could be administered orally as a complete substitute for the hormone of the parathyroid gland in maintaining litter growth and lactation in doubly operated rats. Of course, optimal levels of thyroxine were administered concurrently.

OBSERVATIONS ON MILK PROTEASE

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The presence of a proteolytic enzyme in milk was first reported in 1897. Since that time there have been conflicting reports as to whether the enzyme is a natural constituent or is of bacterial origin. Evidence is presented which confirms the presence of a natural proteolytic enzyme in milk. Using a pH 8.5 borax buffer system which prevented the growth of bacteria, it was shown conclusively that proteolysis was not due to bacterial action. The enzyme survived pasteurization temperatures and resisted higher temperatures before it was completely inactivated. Proteolytic activity was confirmed with two procedures, the Hull tyrosine method and the McFarren buffered filter paper chromatographic method, with relatively the same amount of tyrosine obtained with each method.

OBSERVATIONS ON THE ABSORPTION OF GLUCOSE INTO THE PORTAL VEIN

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In most previous studies, it has been found that glucose is of little or no importance as an end product of carbohydrate digestion in ruminants. This study indicates that glucose may assume importance with some types of rations.

An exteriorized carotid artery and a polyethylene catheter in the portal vein were established in a 500-lb. calf. This allowed sampling the blood simultaneously as it entered and left the intestinal tract. When a ration of timothy hay and corn was fed, the glucose levels of the carotid artery and the portal vein were approximately equal. Using alfalfa hay in place of timothy hay

resulted in significantly higher glucose levels in the portal vein than in the carotid artery. Using portal blood flow data from previous experiments, it was estimated that 10% of the energy of the alfalfa ration could be accounted for by the glucose absorbed into the portal vein. Since carbohydrate absorbed as glucose would provide more energy to the animal than carbohydrate fermented to volatile fatty acids, this observation could assume practical importance.

TECHNIQUE FOR OBTAINING SERIAL SAMPLES OF ARTERIAL BLOOD FROM THE MIDDLE COCCYGEAL ARTERY IN THE BOVINE

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Obtaining samples of arterial blood in the bovine has been considered difficult and often has limited studies in some areas of research.

The purpose of this investigation was to develop a technique by which samples of arterial blood could be obtained at any given interval over a 24-hr. period.

The results of this investigation show that it is possible to introduce a slender tube into the artery on the lower surface of the tail in the bovine and conveniently obtain serial samples of arterial blood over 24-hr. periods without undue stress to the animal.

DEVELOPMENT OF ZINC DEFICIENCY IN HOLSTEIN CALVES FED A PURIFIED DIET

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It has been well established that zinc is a required nutrient for swine, poultry, and other animals. However, zinc deficiency has not been reported previously in any ruminant. In this report a low-zinc purified diet and tap water, containing 2.7 and less than 0.1 p.p.m. zinc, respectively, were used to develop a deficiency condition in two Holstein calves. As has been observed in swine, rats, and mice, the syndrome was characterized by parakeratosis of the skin. Other abnormalities included reduced growth; dull, listless appearance; swelling of the joints of the hocks, knees, and the feet in front of the fetlocks; rough and scaly skin on the rear legs with areas denuded of hair; breaks in the skin around the hoofs; slightly red and inflamed nose and mouth with hemorrhages around the teeth; ulcers on the dental pad; and reduced blood levels of zinc and carbonic anhydrase activity. Two comparable animals receiving the same purified diet supplemented with 43 p.p.m. of zinc did not develop the deficiency symptoms.

OUR INDUSTRY TODAY

THE ROLE OF THE DAIRY TECHNOLOGIST IN EXTENSION[1]

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HISTORICAL

I appreciate your invitation to discuss with you a subject as vital to Extension as the one you have given to me. Dairy technology and Extension methodology have changed with marked rapidity over the years that Extension has played its role in the development of American agriculture. One of my earliest recollections of Extension work prior to the passage of the Smith-Lever Act was a demonstrator from the Dairy Department of the Agricultural College, who appeared at a Farmers' Institute, and who gave a demonstration for the women on butter making.

C. M. Ferguson

There is little doubt in my mind that there was a great need for improved technology in butter making in those days. One of my earliest recollections of playing the role of a dairy technologist was providing the necessary energy to turn a small churn in the summer kitchen at home. My most vivid memory was the day when, in my haste to get the job done, I turned the churn over before my Mother had fastened the top. This was a catastrophe of the first order. A whole week's collection of cream on the kitchen floor was not a matter to be taken lightly, and next week's grocery purchases were dependent in no small degree on the sale of the butter that would have resulted had this young dairy technologist followed instructions a little more carefully. Maybe it was some of these early experiences that convinced me that I was better adapted to be a poultry specialist than a dairy technologist.

The advance of American agriculture from the home churn to modern pipeline milking, from the sale of milk in tin cans by producers on a house-to-house basis, to modern distribution in disposable containers, is one of the great manifestations of the adaptation of science and technology to American agriculture.

FUTURE CHANGES

But, as dairy technology has changed and is continuing to change, so does Extension. The effectiveness of Extension education in the years ahead will be measured in no small degree by the rapidity with which agriculture continues to apply the findings of research and technology in all segments of the industry. I would like to point out that the terms farming and agriculture are no longer synonymous. The agriculture of which we speak is a great complex within our total economy, with its segments extending all the way from the first preparation of the seedbed through crop production, animal care, transportation, processing, packaging, wholesaling, retailing, and consumption. The improvements which have occurred in the last few decades have resulted in the most abundant supply of food of the highest quality for the lowest percentage of the consumers' dollar that we have ever known.

The tremendous rate of growth of our total agricultural industry in this country is due in no small measure to the philosophy of our land-grant institutions, their tireless search for new facts, and a firm conviction that these facts have value only when put to work through education.

Looking ahead, it seems very apparent that we will continue to have fewer farms, larger production units, greater investment, more specialization, more applied technology, more need to bring technology and management into a working combination, and more dependence by the producer on a highly developed distribution system.

The dairy industry, like other segments of the agricultural economy, will continue to meet stiff competition. The demand for improving quality will go on unabated. Changes in the distributive system will often be costly, and obsolescence of equipment and distributive methods will continue as the fruits of success continue to go to the innovator. The consumer of tomorrow will be a much more intelligent buyer than the consumer of today or her counterpart of yesterday. She will continue to demand more and more built-in service. Her knowledge of nutrition and her insistence on putting this knowledge to work in preparing everyday diets for her family will continue to sharpen the thinking of producers, distributors, researchers, and educators.

GUIDES FOR THE FUTURE

Against this backdrop we see Extension continually evaluating its program and its methods. We see more and more emphasis being placed on the development of decision-making skills, not only by farm families but by those responsible for the processing and distribution of farm produce. The problems of surplus are focusing attention on both research and education in the field of utilization. No segment of our industry can rely on technology alone

[1] Presented to the Dairy Manufacturing Extension Section at the 55th Annual Meeting of the American Dairy Science Association, June, 1960.

to answer all of its problems. Those who guide its future must be concerned with public affairs and the many economic and social changes which bear on our everyday life. Extension, in attempting to set some guideposts for the years ahead, developed as a national guideline the Scope Report. This document identified nine areas of emphasis. It pointed out the significance of Extension's effort to improve efficiency in marketing, distribution, and utilization, as well as in the production of agricultural products. The emphasis that the Scope Report placed on management on the farm and in the home, and on family living, has a direct bearing on the production and ultimate use of agricultural products in the maintenance of a healthy populace. Extension work, being an educational effort, relies on the change in the knowledge and skills of people. The significance of that section of the Scope Report dealing with leadership development also has great meaning to subject matter specialists. The results of our work will be measured in no small way by the effectiveness of those with whom we work, as they accept the responsibilities of leadership within the community or within an industry.

The Scope Report has been criticized by some because it represented an effort on the part of Extension to look at itself. Some felt a report of this kind should have been written by a selected group of Extension's clientele. I would be the first to defend the Extension Service, or other public agencies, in its effort to examine its own objectives, its methods and its accomplishments, but I would be equally enthusiastic about the development of ways and means of encouraging our many publics to also take a critical look at our efforts and evaluate them.

Each of our publics tends to look at us in the light of their particular interests. Extension administrators find themselves under constant pressure from special interest groups for specialist assistance, while others are inclined to place less emphasis on the role of the specialist and more on the role of the generalist.

SPECIALISTS FOR THE FUTURE

With increasing demand for specialized help, I would predict that we will see staffs of the future composed of more specialized agents and even more specialized specialists.

This brings us to a consideration of the role of the dairy technologist who, of course, is a specialist. What kind of an image do your administrator, your colleagues, and your many publics have of you and your role? This question may be too broad to be easily answered, because each of these groups, depending upon their particular interest, may have a different image of you, your work, your responsibility, your program, and your accomplishments. Maybe we can examine some of these briefly. I believe there would be general agreement that one of the roles of the specialist is to provide a bridge—a two-way street—to insure rapid, accurate communication between the research worker and the departmental executive on the one hand, and on the other, those who are actively engaged in dairy manufacturing and distribution. This two-way street will not be an effective thoroughfare unless the specialist finds effective ways of helping industry personnel analyze their problems, apply the appropriate technology, but in turn reveal to the researcher the voids which exist in our knowledge which need to be filled by further research in order to find answers to the ever-changing problems of the industry.

MARKETING SPECIALISTS

Some concern has existed from time to time in the role of the marketing or utilization specialist in his relationship to the county staff. Last year, a study was undertaken by the National Agricultural Extension Center for Advanced Study at the University of Wisconsin, in an attempt to sharpen our thinking in regard to off-farm extension work, particularly in the field of marketing and utilization.

The following quote from this report bears on this point, and is being given consideration by Extension administrators and program leaders:

"Develop policies that permit marketing personnel to work directly with marketing firms as well as through county Extension offices.

"County agricultural agents, although sometimes interested in marketing work with farmers, have not and will not in the future, in most cases, have the time, training, or experience to work with marketing firms, particularly those beyond the first assembly level. The demands for the time of county agricultural agents by producers, with their need for increasingly specialized assistance, are likely to increase rather than diminish. It does not appear reasonable to expect county agricultural agents to become proficient in the increasingly complex problems of marketing as well as of those in agricultural production. Every agent, however, should be kept fully informed of Extension marketing programs with marketing firms in his county or those that affect producers in his county."

COOPERATION BETWEEN DISCIPLINES

There is a growing consciousness among specialists and extension administrators for the greater need for collaborative approaches, because so many problems in an industry or within an enterprise of necessity cut across several subject matter areas. For instance, the matter of quality milk can not be considered thoroughly without adequate consideration being given to agronomy, to feed quality, to agricultural engineering, to bio-chemistry, bacteriology, and economics. The specialist today is finding a growing demand for his services

as a team player, and his effectiveness on the team calls for the ultimate in his knowledge and familiarity with the several disciplines which bear on the subject matter for which he is responsible.

The need for interplay between subject-mater areas is being recognized by extension administrators. It is being revealed in current discussions on the consolidation of project agreements, which are one of the formal instruments of cooperation between the Department and the land-grant colleges. Present effort is in the direction of molding of project agreements in contrast to the trend some years back, which was one of splintering.

DAIRY EXTENSION TECHNOLOGIST

Let us look, then, at the role of the dairy technologist—

1—as a competent specialist and authority
2—as an educator and a leader
3—as a member of the team

Certainly, the central purpose of the teaching of the dairy technologist must be to contribute to the growth and competence of those with whom he works. Only through such growth by those in the industry will it be successful in more efficient processing, distribution, and the continual upgrading of quality and nutritional value, as well as the development and adoption of new and improved products. The core groups to which the dairy technologist directs his educational effort are the processors and manufacturers. His knowledge of technology is also needed by firms who manufacture and supply equipment and, certainly, his audience from time to time must include the producers whose investment in milking, cooling, and holding equipment continues to grow, and on whom there will be a constant pressure to produce a finer product.

The dairy extension technologist as a teacher follows the same sound educational techniques any other extension educator finds productive. He must discover ways to help members of the industry to identify and analyze their problems such as low quality, processing deficiencies, inefficient plant design, plant management limitations, consumer relations. Having done so, he must then develop a program using proven educational methods to bring appropriate research findings to bear on specific problems.

This puts the spotlight very sharply on the need for the specialist to be in constant contact with his counterparts in research. While his most intimate personal contacts will be with his own experiment station research workers, he must, through the literature, maintain a constant contact with the work being done in other experiment stations, in the agricultural research laboratories of the Department of Agriculture, in such agencies as the Agricultural Research Service and the Agricultural Marketing Service, and in industry.

The dairy extension technologist is most effective when he maintains close working relationships with state and federal agencies having the responsibility for regulatory and health programs, and a close liaison with trade groups in the dairy field can pay good dividends. Here, the most appropriate relationship is that of serving as a resource person, as a consultant and an advisor, rather than becoming too deeply involved in the time-consuming and often association-promotion activities associated with the role of secretary.

On the extension team the dairy extension technologist has a key role to play as he works in consonance with dairy production specialists, nutritionists, specialists in consumer education and dairy marketing, as well as with the engineers and economists.

The greatest challenge in extension education today is to build with the full support and backing of our interested publics a well-coordinated program. Such a program is most effective and produces the greatest satisfaction for those who are responsible for it when all disciplines wherever found are marshalled in such a manner that all segments of the industry move forward. It calls for a full measure of sound educational engineering.

MAMMOTH CHEESE

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For some years, the Historian of the American Dairy Science Association has been fascinated by stories on big or mammoth cheese and has made a collection of some of them. Thinking perhaps that they should be documented for the enlightenment and possible delight of a newer generation, the review herein was written.

Stories on mammoth cheese excite the imagination. Often, extravagant claims not supported by facts are made. These stories are excusable because memory lapses, few records are available, and the cheese at hand truly is a monster. As such, bigger-than-standard cheese arrests attention. All these stories are most interesting, for they not only portray the ro-

mance surrounding early cheese making but stir the imagination concerning the labor and raw products involved in creating a stable form of milk which keeps without refrigeration.

Frequently, a distinguished personality in any industry, particularly in the various branches of the dairy industry, is referred to in slang terms as the big cheese. Recently, Block (1) explained the history of thus titling the head man, as an old American practice of honoring the nation's head man with a gift of the biggest cheese available.

According to Block, the custom of honoring the President with a gift of a cheese began in 1801, when some Connecticut farmers presented President Thomas Jefferson with a 1,235-lb., 4-ft.-in-diameter cheese made from a day's milking of 900 strictly Federalist cows. Later, Andrew Jackson was the recipient of an even bigger cheese, weighing 1,400 lb. Time seemed to have shrunk the cheese, for in 1928 Calvin Coolidge's presidential cheese was a puny 147-pounder.

Nonetheless, narrated Block, monsters had been made meanwhile, culminating in the all-time greatest at the 1911 Chicago National Dairy Show. This Cheddar mammoth stood 5 ft. high, had a diameter of 8 ft., and weighed 12,361 lb., or more than six tons. Perhaps this not-so-little specimen was created out of time and place for getting attention. The World's Fair, of all fairs, was less than a score of years past. The wonders of the Chicago, 1893, Columbian Exposition, including the Ontario Cheese display, were still fresh in the memory of thousands. Guthrie (8), dairy processor, attended the National Dairy Show in Chicago in 1911, but had no recollection of the mammoth cheese on display at that time; at any rate, no additional information about this cheese was found.

ONTARIO'S OUT-SIZE CHEESE

Murphy (12) reported several accounts in Canadian writings on mammoth cheese. He believed that those of Dr. J. A. Ruddick, four-decade Dairy Commissioner of Canada, commonly known as the cheesemaker, were of unusual historic value. Mr. Ruddick wrote that cheese of abnormally large size had always excited considerable interest, and that the idea of making such cheese appeared to be a fairly old one. His brief account of the Jefferson cheese is slightly different from that of Block's. It follows:

"In 1801, the people of Cheshire, Massachusetts, made a cheese which was said to have weighed 750 pounds, and presented it to President Thos. Jefferson. In 1836, one Col. Mecham, of New York, presented Andrew Jackson with one that weighed 1,400 pounds."

While the Jefferson and Jackson cheese seem small compared to today's giants, one must recall that all cheese was farm-made prior to the introduction of the factory system of cheese making by Jesse Williams in 1851 (18).

The Canadians took an early interest in making large cheese. Ruddick reported that before 1860, Hiram Ranney of Salford, Ontario, made several large cheeses in his farm dairy. One weighed over 1,200 lb. The making of such a large farm cheese was no little job. Conservatively, the curd must have required 12,000 lb. of milk, or about 1,150 Imperial gallons, an amount at that time far beyond that of a single farm production.

The introduction of the factory system of cheese making soon widened the horizon of the cheese maker, so far as big cheeses were concerned. According to Ruddick, Andes Smith, in 1865, made a cheese weighing 4,000 lb. in his new factory near Norwick, Ontario. This cheese was displayed at the New York State Fair.

Smith's up-to-that-time-unheard-of 4,000-lb. Cheddar created quite a stir among the New York, Herkimer County, cheese makers. On this, Biggs (3) furnished the following interesting sidelight taken from the Toronto *Globe*, September, 1865:

"The chief feature of the New York State agricultural show last week was the monster cheese from Canada. . . .

"The Herkimer county farmers felt that Canada had beaten them and vowed with many strange asservations that next year they would show what their county would do in the way of a big cheese.

"The visitors to the show exhibited no mortification over the matter. They seemed to be well pleased with the Canadian display and perhaps guessed that if the Canadians made such large cheeses they would have to be annexed whether they liked it or not. . . .

"Seriously speaking, the promptitude with which our Canadian farmers have taken up the idea of establishing cheese factories on a large scale is exceedingly gratifying, and an earnest of good for the future prosperity of the country.

"Not only is cheesemaking exceedingly profitable, but the fact shows life and enterprise among our farming community which will bear fruit in other matters, as well as cheese."

Not to be outdone in out-size Cheddar manufacture, Mr. Ranney, champion large-cheese maker of the fifties, in 1866 joined forces with his son-in-law, James Harris, of the Ingersoll, Ontario, cheese factory and turned out a 7,000-pounder. This cheese was exhibited both at the Saratoga New York State Fair and the Kingston Provincial Exhibition that year (16).

Apparently, this whopper put an end to big-cheese making competition or else by then such cheeses were taken for granted. For a score of years nothing of significance in big cheese making seemed to have been recorded. Then again, the big ones were produced in increasing numbers. Ruddick records that during the

eighties, James Ireland, of the Galloway, Ontario, factory made 35 large cheeses, 11 of which weighed 5,500 lb. each. The others ran from 600 to 1,000 lb. each. Between 1886 and 1895, D. M. Macpherson, Lancaster, Ontario, turned out almost 100 cheeses weighing from 1,000 cheeses were used for show-window purposes to 1,200 lb. each. Interestingly, all of these in the United Kingdom.

RECENT MAMMOTHS

In all fairness to Block (4), and to put the record straight, mammoths of recent vintage have actually outweighed the Chicago National Dairy Show 12,361-lb. cheese. Within the past 2 yr., at least two mammoths have made the news columns. In 1958, the Cincinnati Junior Chamber of Commerce (10) brought The World's Largest Cheese, supplied by Kraft and made by Siudzinski (15), to Cincinnati for the September Food Fair (Figure 1). This cheese was described as a seven-ton Cheddar.

More recently, Steve Siudzinski, Denmark, Wisconsin, made the cover of the *Milk Products Journal* (11) with his 13,913-lb. behemoth. According to Siudzinski, the modern master of the mammoths (Figure 2), this size is about the limit, as it fills the doorway to the cooler.

Statistics on this giant bug the eyes. The whopper was 8 ft. in diameter and 8 ft. in height. This attention-arrester required 146,086 lb. of milk, or 67,947 qt., which at 20¢ per quart would have cost $13,589.40. Into this milk and resulting curd went 150 oz. of vegetable cheese color and 400 lb. of salt. The curd was made in ten 15,000-lb. vats in three factories. Twenty-two cheese makers and helpers worked on this cheese, which required 38 sq. yd. of cheese cloth, 15 sq. yd. of press cloth, and 68½ lb. of paraffin to cover it. Six

FIG. 1. A recent seven-ton mammoth displayed by the Cincinnati Junior Chamber of Commerce to promote a food fair (15).

FIG. 2. Steve Siudzinski, Denmark, Wisconsin, amidst some mammoths of his own make (7).

hundred and fifty board feet of lumber were used to make the hoop for pressing the curd and platform on which the cheese was moved about (17).

Siudzinski began making the big ones in 1934, the sizes ranging at that time from 1,000 to 5,000 lb. (11, 13). Since then, the range has extended to nearly seven tons. These monsters were used by grocery stores and supermarkets across the nation in gigantic promotions designed to attract the attention of the entire city. The one displayed at a Dayton supermarket received wide acclaim (9). As an example of the success of such promotion, the *Milk Products Journal* (11) reported as follows:

"Last spring the Liberal Markets of Dayton, Ohio, decided to center a store promotion around a mammoth and purchased a 13,000-pound cheese from Steve, also through the Kraft Foods Co. The promotion was held for a three-fold purpose: to get Daytonians to associate good cheese with Liberal Markets; to increase local cheese consumption, and to draw new customers into the store.

"The two-week promotion proved even more successful than Liberal's staff had imagined. Results for the first week at the firm's downtown store showed an increase of 76.7% in dairy sales, 19.8% in store traffic, and 13.6% in total store sales. The second week showed a dairy sales increase of 45%, a traffic increase of 13.8%, and a total store sales increase of 10%. Figures for the week following the promotion showed a 6.5% increase in dairy sales, a 3.7% increase in traffic, and an 8.6% increase in total store sales."

From the above, the point must be made that the mammoth cheese is marketable as natural Cheddar. On this matter, Siudzinski (17) writes, in part, as follows:

"We have sometimes been reminded that there have been others who have made larger cheeses than we have. While this is true, we still maintain that we make the world's largest market-

able cheese. That is, our mammoths can be paraffined and cured for as long as 1½ to 2 years and longer. . . .

"Our mammoths carry the Wisconsin State Brand clearly imprinted on them, which signifies that they are of highest quality and texture."

A QUEBEC GIANT

An 8-ft. Cheddar " géant" displayed at the Quebec Provincial Exposition from September 4 to 12, 1953, and at the Canadian Dairy Industry Show, Montreal, October 23 to 27, literally awed thousands of visitors (14). This dream project of Camirand (5) was most effective in calling attention of the Quebec population to the important role which the dairy industry played in Quebec's economy. Subtly, the monstrous cheese refreshed the memory of all concerning the great nutritive value of dairy products, particularly cheese, in the daily human diet. In addition, this huge bulk of taken-for-granted dairy product served to promote the best dairy interests in that section. Probably of most lasting value and least suspected was the lift it gave both dairy producers and processors, leaving them with a priceless pride in their own industry.

This five-ton giant was the product of 100,000 lb. of milk from 4,000 cows owned by 400 producers, and required 442 man-hours of labor. Sixteen cheese factories contributed the curd. The cheese made according to modern technique was reported to have had a quality comparable to the best Quebec Cheddar.

A feature of the striking mammoth cheese display at the Quebec Exposition, attended by about 400,000 annually, was the weight-estimating contest for which substantial prizes were offered. In justice to all, several persons worked several days to examine the enormous number of responses and to determine the winner. Strangely, eight persons estimated exactly the weight of the cheese—10,960 lb.

Conservatively speaking, few industry-wide promotional enterprises have surpassed the Quebec Cheddar "géant" project in accomplishing its avowed purpose of bringing favorable attention of producers and consumers alike to the important role that agriculture plays in the economy of a nation. Other cheeses have excelled in size, but it is doubtful if the concentrated, pin-pointed promotion directed to an area audience has ever been equaled.

THE ALL-TIME, REALLY BIG, 1893 COLUMBIAN EXPOSITION MAMMOTH

In keeping with the extensiveness of the Dominion, the Canadians seem to have had a rightful yen for outsize agricultural product display. The talk of the 1893 Chicago Columbian Exposition (Figure 3) was the Canadian mammoth cheese (Figure 4), the central motif of the Province of Ontario display, which depicted the scope of its cheese industry. Bancroft (2) in The Book of the Fair briefly records this display as follows:

"Of cheeses the making of which—a prominent Canadian industry—there is a sufficient display, and one of the chief attractions in this section is a monster cheese, encased in an iron tank, and mounted on a platform approached by a flight of stairs. On an inscription attached to the stand we are informed that it weighs 22,000 pounds, and that to supply the materials more than 1,600 maids milked 27,000 gallons from 10,000 cows."

Ruddick (16) gives a fuller description of this titanic of the Cheddars. His comments follow:

"The largest cheese on record was made at Perth, Ontario, in September, 1892, and it was shown at the World's Fair, Chicago, in 1893. The special press for this cheese was set up in the Canadian Pacific railway freight shed at Perth station. At the proper stage, on the appointed day, the curd from twelve cheese factories was carried in milk cans to the station and dumped into the hoop, which was made of ¼-inch steel boiler plate. It required two days' curd from the twelve factories and a further supply on the third day from three of them to fill the hoop. The curd was pressed after each filling, twelve large jack screws being used for the purpose. A pressure of more than 200 tons was applied.

"The following particulars are taken from a leaflet distributed at the Fair:

" 'The Canadian Mite' was manufactured at the Dominion Experimental Dairy Station at Perth, Lanark county, Ontario, under the supervision of Prof. James W. Robertson, Dominion Dairy Commissioner.

" 'The total quantity of milk used in making this cheese was 207,200 pounds. That quantity

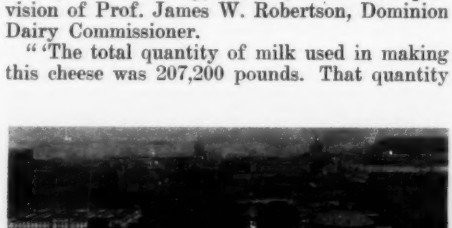

FIG. 3. Bird's-eye view of the Columbian Exposition, Chicago, 1893, where the world's largest cheese, 22,000 lb., was displayed by the Province of Ontario, Canada (2, 6, 16).

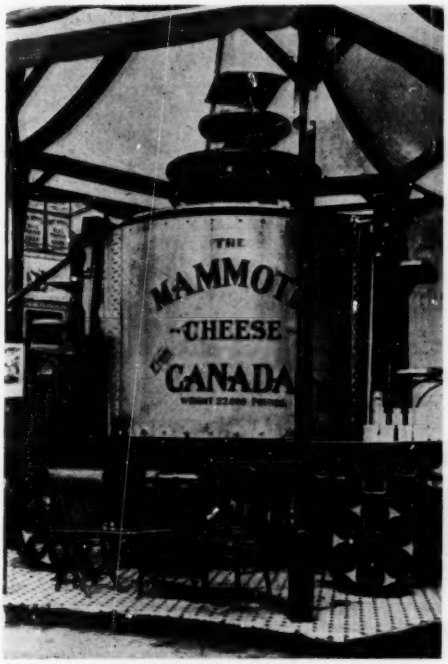

FIG. 4. The Ontario mammoth cheese shown at the Columbian Exposition, Chicago, 1893 (2).

is equal to the milk for one day in September of ten thousand cows. Mr. J. A. Ruddick, of the Dairy Commissioner's staff, was the cheesemaker, and he was assisted by cheesemakers at twelve adjacent factories. . . .

"'The cheese weighs 22,000 lbs. net. It is incased in the mould or hoop of steel in which it was pressed. It measures twenty-eight feet in circumference by six feet in height.

"'A special truck has been made for transporting it through Great Britain after it leaves Chicago. It has been sold to Mr. T. J. Lipton, of London, England.'"

Mr. Lipton, later becoming Sir Thomas, failed to carry out his part of the contract, so the cheese was finally shipped to A. J. Rowson, London, who disposed of it to a well-known caterer. In May, 1894, a large piece of it was returned to Ottawa, where it was found to be in excellent condition (16).

QUALITY OF THE COLUMBIAN EXPOSITION ONTARIO MAMMOTH

With the passing of time, considerable speculation has occurred as to the edible quality of this huge hulk of Cheddar. Cheese makers readily visualize that a 22,000-lb. mass of curd is equivalent to 1,000 standard-size, 22-lb. daisies. If the cheese were of marketable quality, 1,000 grocers could dispose of these daisies effectively within a reasonable period of time. But the marketing of a 22,000-lb. monster is a cheese of another color!

How did this immense steel cylinder of curd finally cure out? No data were found relative to the curing of this cheese. Only speculation remains. One must be reminded that the mammoth stood for 6 mo. under the glass-roofed exhibition building, where it was exposed to great summer heat. One could marvel that, under these conditions, the curd did not blow up. However, a point often overlooked when speculating about the adverse conditions to which the 1893 World's Fair cheese was exposed for curing is the fact that the cheese was made in September, 1892, but that the Columbian Exposition did not open until the spring of 1893, a lapse of 8 mo. Considering the probability that 1 mo. would be required to transport and place the mammoth in exhibit, seven critical curing months yet remained for controlled ripening of the cheese. By the opening of the Exposition the bacteria would have gone through their greatest state of proliferation. Likely, no further or adverse bacterial growth would have occurred during the hot summer exhibit days of 1893. This probably explains, in part, why the cheese was of such high edible quality as it was reputed to have been.

Documented evidence reveals that the cheese did withstand the rigorous unorthodox ripening period, was of good quality, and finally was marketed for human consumption. Lipton of England and of tea fame evidently saw advertising possibilities in this cheese and contracted for it.

Another factor pointing to the imperativeness of producing and maintaining edible-quality mammoth cheese, which must not be overlooked, was the nature of the market for natural Cheddar Cheese in 1893. This mammoth was made and exhibited before the days of processed cheese (20). Grinders and blended cheese were not in the cheese makers' vocabulary at that time, let alone in their thinking as to possible market outlets. Cured cheese was sold for human consumption and that was it.

Undoubtedly, price was a factor which enabled cheese makers and food merchants to dispose of inferior-quality cheese. Probably very little, if any, Cheddar Cheese was destroyed or sold for animal feed in those days. Then, as now, Cheddar Cheese was a highly rated, concentrated stable form of milk, too precious in price and in nutrition to ruin through neglect of proper care and cure.

OUT-SIZE CHEESE A FAVORABLE ADVERTISING MEDIUM

A review on mammoth cheese would be quite incomplete if mention were not made of the role of big cheese in advertising. Let it be said that a complete revolution in cheese merchandising has occurred during the past two-score

years and that consumers are more cheese-conscious today than ever before. Spectacular achievements in cheese consumption have been attained. The United States per capita consumption of cheese has virtually doubled during the 40-yr. period from 1916 to 1956 (19). Undoubtedly, this steady increase in the use of cheese can be attributed, in a large part, to (a) changing food habits in the American dietary; (b) improved quality; (c) development and introduction of processed cheese; (d) attractive packaging; (e) extensive advertising; and (f) effective merchandising. Of these factors, advertising, news stories, colored magazine advertisements, radio, and television have played no little part.

The development of the supermarket has made possible a unique phase of cheese advertising embodying in several respects the spectacular, the drama, and the suspense which one sometimes erroneously associates with early circus advertising. This advertising often centers about the larger-than-usual sizes of American Cheddar Cheese. Generally, the cheese itself is referred to as a mammoth.

Such means of calling consumers' attention to cheese seems trebly effective. In the first place, consumers today, familiar with retail packages, rarely see cheese in its uncut state; secondly, the size of the cheese can be dramatized in terms of the number of cows, pounds of milk, or man-hours of labor required to produce it; and thirdly, consumers like to watch tne actual preparation of food for sale, such as cutting and wrapping of cheese. Particularly is the latter effective when sampling is permitted. That this method of enticing customers into stores and selling cheese as well as other foods is effective can not be denied.

THE SONG OF THE MAMMOTHS WILL CONTINUE

This brief résumé on some spectacular cheese of history is not complete without reminding ourselves of man's inner urge to outdo that which has been done in the past. One may predict with no little confidence that the mammoth cheese of yesterday will become the Goliath of tomorrow. Although data on some future world's largest may not withstand the searchlight of history, the misclaims in themselves really are inconsequential. This statement would seem to be inconsistent with scientific principles based upon truth. But such claims are relevant. They serve their purpose.

Actually, the rhetorical bigness of the cheese can be measured only in terms of its influence upon man's imagination and its effect upon his reactions. Past mammoths with accompanying statistics and claims to bigness have stirred the imagination, given a sense of pride to an industry, awakened consumers to a realization of their dependence upon others for their daily bread, and have subtly created an awareness that good-eating enjoyment yet remains with the simple, basic foods, such as cheese.

Romance enshrouds few foods so lavishly with folklore and history as it does cheese. One standing before the cheese market—or beside a big cheese—can be easily wafted away to distant lands as he allows himself to dwell momentarily upon the making of the dairy industry's second oldest and most widely used product of milk, cheese—a product sought after down through the ages by peasant and potentate alike. The glamour of cheese is interwoven (a) with the folklore of nations, (b) with the economic welfare of peoples, (c) with international politics, and (d) even with world history. Gourmets have long prided themselves in knowing how to select, serve, and enjoy cheese to its fullest.

Widely acclaimed and honored as milk's unmatched concentrate, cheese, developed and ripened to suit the palate of the most discriminating, always makes an attention-arresting display. Little wonder that modern supermarket cheese counters have cash-register appeal.

The giants symbolize all cheese—not Cheddar alone. Through their display the consumer is enticed into an unexplored world of cheese. Here he may wander into a new wonderland and see with Baron Munchausen that, "Upon this island of cheese grows great plenty of corn, the ears of which produce loaves of bread, ready made."

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ROLE OF SHORT COURSES, CONFERENCES, AND NEWSLETTERS IN PERSONNEL TRAINING [1, 2]

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Each department must adopt a philosophy and develop appropriate adult education or continuous educational programs to help industry educate and develop men while the men are being employed. These should be organized in accordance with the desires and need of the commercial industry. These are of several basic types:

M. E. Gregory

(1) *Short courses*—2 wk. or more—full-time; to train men how to do the job better in plant work. Some departments are very successful with them. We had moderate success for a few years. Two years ago, after surveying industry, they were discontinued because of lack of interest. One of the main problems was that they are designed for small plants, but small plants can not spare key personnel full-time for 2 wk.

(2) *Short courses*—correspondence. We introduced a 5-mo. correspondence short course 2 yr. ago which involved weekly lessons for those who participated and a one-day visit at the university per month. This was unusually successful and the response on the part of those who took the course and from the management itself was unusually good.

The Problems: (a) The tremendous work involved for the staff; (b) the lesson material may be used by a large number of persons in a plant—other than those enrolled in the class. This makes unpredictable the success of the course in subsequent years. However, a 1-yr. trial of this course by our department revealed tremendous possibilities.

(3) *Conference:* A 2-4-day refresher conference. This is a most effective way for a department to serve industry. Some of these are designed for a specific subject, such as bulk tank handling. Some are designed on an annual basis, to serve the entire industry. Our particular conference is a three-day conference which is so organized as to serve all branches of the industry in Ohio. We have found it to be unusually successful, and we consider it to be the high light of our departmental program.

We have found that the conferences need to be constantly scrutinized and the subject matter presented must be examined in terms of what it will do for the people themselves. The material must be up-to-date and down-to-earth. It must help the man do a better job.

One development in this connection is to place extra stress on management. Although the technical phase continues to be important, we found that the registration was going down because management itself was not aware of the value of this program. The management section which was incorporated 2 yr. ago proved unusually successful.

Conclusion: The conference is a specific teaching aid for the commercial industry and a means by which tremendous benefit can be derived by those who attend.

Adequate communications is a key to an effective department-industry relationship: Too often a definite breakdown occurs between the department and the commercial industry because of lack of understanding on the part of the industry as to what the departmental problems are and what the industry may do to help solve them. This failure of understanding is a result of poor communications between the educational branch of the industry and the commercial branch, and the major responsi-

[1] Special appreciation is expressed to Dr. I. A. Gould, Chairman, Department of Dairy Technology, Ohio State University, whose earlier presentation before the Sealtest Council served as a basis for this presentation.
[2] Presented to the Dairy Manufacturing Extension Section at the 55th Annual Meeting of the American Dairy Science Association, June, 1960.

bility for this must rest with the department Therefore, a department must use every means at its disposal to attempt to bring understanding to the industry as to what the department is doing in the various areas of education, research, and service. This may be accomplished in various ways:

1. By short courses and conferences (indicated above).

2. By working closely with the dairy technology societies. There are four societies in Ohio and a staff member of the department serves as corresponding secretary of each of these societies. It is his job to serve as a liaison representative between our department and these groups.

3. By projecting the staff into industry problems within the area. In Ohio, we have the privilege of serving as a member of the board of the Ohio Dairy Products Association and, in this capacity, take advantage of every opportunity to present views in respect to the department at the various board meetings.

4. To meet with industry groups within the area wherever possible, in order to have them understand some of the problems in which both the department and the industry are concerned. One such meeting occurs annually, in connection with the State Association Convention, where a sectional meeting on dairy education has been held for several years, at which time the entire matter of scholarship programs, high school counselling and recruitment, etc. are discussed.

5. By written releases carrying information and suggestions to all branches of the industry. This is a most effective way, since many people are contacted at one time. Our department uses the following:

The Dairyologist—a quarterly news release which goes to alumni and dairy plants alike, dealing with the various activities of the department, the university, and the industry.

Research Digest—a brief summary, written in lay language, of some pertinent research finding or dealing with some particular problem which has been brought to the attention of the staff, and which is being experienced by someone in the field. It may deal with the matter of cream feathering, with poor setting of cheese, or with a variety of other matters. Individual staff members assume responsibility for preparing these in coopeeration with the extension specialist, who distributes them throughout the state.

Scoops—a one-page release dealing with some current matter of particular interest to fieldmen. It is prepared in such a way that, in many cases, the fieldman orders a large supply of them to be distributed directly to producers. It may deal with some problem of malty flavored milk, the cleaning of milking machines, recommendations on antibiotics, or a variety of other subjects. Again, it is designed to have the copy effective and understandable so that the message can be gotten across quickly. This release is prepared by the specialist.

Epistles on Dairy Technology—a personal letter to the dairy plant manager from the department head. In this case, the letter is sent directly to the dairy plant manager and only a selected number of these persons receive the communication. It deals with timely topics, or perhaps the philosophy that involves management problems.

Efficiency Tips—this is the latest member of our regular communications. It is designed to be of particular interest to middle management. Management techniques are described and demonstrated, such as production scheduling, job evaluation, standard times, etc. The purpose of such is to promote the use of these techniques in dairy operations. This release is prepared by the specialist.

Subject matter pamphlets and field bulletins—dealing with particular problems of concern to the plant operator, the employee in the plant, or the producer. Examples are pamphlets on fat control, coliform control, laboratory counts, etc. Field bulletins are articles that have been reproduced and distributed by the specialist. They usually are on some timely subject, and merit circulation.

Service: As indicated earlier, one main phase of any department's work is that of service. In the previous section on communication and gaining understanding, many references are made to service activities of the staff and the department.

As a further word, it may be said that the department can function in an effective fashion by having an open-door policy from the standpoint of serving the industry and helping it solve its various problems of management and operations.

In many states, the dairymen do not realize the tremendous services they can obtain from their own dairy department, but in other states the dairymen are generally making full use of this opportunity. Here again, communications and understanding play a role.

The department can, through its effective communication system, bring to the attention of the dairymen, both large and the small, that the department is there to serve. Then maximum use will be made of the department and of the staff by those who need assistance.

Conclusions: All of the above point out the various means by which the department can serve the industry. There are other ways which are not indicated. There are many details of these broad programs which could be dwelt on to a large extent. However, the department of today must achieve greater support than ever before from the commercial industry, if it is to continue as an integral part of our advanced educational system.

To justify the active support of industry, the department must play its major role in:

1. The recruiting, educating, and developing of the college-trained man.
2. In organizing and carrying forward effective adult educational programs in cooperation with industry.
3. In achieving an effective means of communication with industry, so that researches and information are supplied quickly and so that full understanding may prevail between the commercial industry and the department.
4. By making its staff and facilities available to the industry in the solution of individual problems.

The department staff can no longer dwell in the ivy-covered tower. The department must become an integral part of the entire dynamic dairy industry.

ADOPTING AND USING A DAIRY PLANT TRAINING PROGRAM[1]

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The greatest asset of any dairy plant is its personnel. Of even greater value than the name and reputation of the company is the organization that made the name and reputation for the company. Few people, other than those in top managerial positions, realize that their organization has no corner on the market when it comes to the purchase of raw products, supplies and materials, equipment or even the construction of a building. All physical assets of a company can be duplicated by anyone who may desire an organization with the same physical features as that owned by someone else. Consequently, the only item a plant has about which it can really boast is its personnel.

H. B. Henderson

Recently, I was in a plant that prints a guarantee on the side of its milk carton giving the consumer assurance that the contents of the package will keep for at least ten days. That was high quality milk. But what made it high quality? The cows? Yes, but with the help of a good farmer who was concerned about the health of the cow, the cleanliness of the equipment, and the rapid and efficient cooling of the milk. Perhaps the type of equipment had something to do with the high quality of the product? No doubt about it. But even more important than the type of equipment are the men who operate it. They, too, must be interested in seeing that the equipment is properly cleaned and sterilized, that the proper amount of heat is applied, that the homogenizer is operating correctly, that the milk is properly cooled, packaged, stored, and delivered to the consumer. The guarantee on the package was not made possible because the plant uses a certain brand of cleaning materials, or a certain brand or type of carton, or because the equipment carries a certain manufacturer's label. Only the interest of all the people in the organization in producing a high quality product made this guarantee possible.

As an industry makes progress, it becomes increasingly difficult for it to make further progress. In breeding dairy cattle, for example, it is not difficult to show marked improvement in the production of one generation over the previous, if the beginning was at a low level. As production increases, however, it becomes more difficult to select animals that will continue this upward trend. As I look back on my first Dairy Industries Exposition 30 yr. ago, I thought at that time, and so did everyone else who saw it, that this was the ultimate in dairy equipment. But now, if one could reproduce the Dairy Show held in Cleveland in 1930, it could be advertised as the greatest antique show on earth.

It has taken men with imagination and vision, to say nothing of their willingness to risk much, both within the industry and without, to continue the development of equipment for the dairy industry. But this constant improvement in equipment has also made it necessary for management to pay closer attention to the selection and training of personnel to operate the plant.

During the period following World War II, when jobs were very plentiful and many industries and professions were replenishing their personnel, many recent graduates in dairy technology were leaving the dairy industry for other jobs. Very soon this was reflected in a marked drop in enrollment in the dairy departments over the nation. The industry leaders became concerned, and the Milk Industry Foundation decided to conduct a survey to determine the reason for the drop in enrollment and decreased interest of college graduates in employment in the dairy industry.

It was my privilege to assist with that survey and to visit with colleges, industry leaders, and college graduates. The survey revealed many factors which influenced the decision of college graduates to leave the industry for

[1] Presented to the Dairy Manufacturing Extension Section at the 55th Annual Meeting of the American Dairy Science Association, June, 1960.

other professions and industries. You are aware of some of these. Such conditions as long working hours, low rates of pay, poor working surroundings, lack of recognition by management, promotion into management of others less qualified, etc., were mentioned many times. But the item mentioned more often than any other, and the one emphasized the strongest, was the lack of a good training program in dairy plants.

Because of the publicity given the results of the study, the Executive Board of the American Dairy Science Association proceeded to set up a committee to develop an outline for a training program. It has been my privilege to serve as chairman of this committee. The committee members were very carefully chosen from colleges as well as from industry. If one person could be singled out as having made the major contribution to the work of the committee, that individual would be Dr. Ira Gould, of Ohio State University. In addition to Dr. Gould, special credit should go to Mr. Farnum Gray, of the Southern Division of Sealtest Foods, Charlotte, North Carolina, and Dr. F. C. Ewbank of the Michigan Milk Producers Association, Imlay City, Michigan. Other industry leaders who made noteworthy contributions were Mr. H. J. Williams of the Carnation Company, Los Angeles, California, and Mr. Newton Lamb of the Borden Company, Houston, Texas, who later withdrew from the committee. However, the distinguished Secretary-Treasurer of the American Dairy Science Association, Dr. H. F. Judkins, because of his experience both in education and in industry, was able to make a most valuable contribution to the development of the outline. He was a great stabilizing influence in all of the deliberations of the committee. His enthusiastic support of the project served as a constant stimulus to the other members of the committee.

As a result of the work of this committee, the American Dairy Science Association recently published a Dairy Industry Plant Training Manual. This manual is so designed that it can serve as a guide in the development of a training program in any dairy plant, regardless of its size or type of operation.[1]

May I emphasize here that this manual was not prepared as a college textbook. Some schools, however, are using it successfully as a reference book in dairy plant management and other dairy technology courses, to familiarize their students with the book before they begin a training program in industry. It has been of interest to note that many plants are finding that present supervisors are using the manual to good advantage for their own personal improvement and as an operating guide in plant procedures. This is an added benefit which the committee did not visualize at the time the manual was being developed.

The Manual, like all Gaul, is divided into three parts. Section I, the introduction, was condensed from about 15 pages, originally, to less than two pages. This was done in an effort to include no more material than management would take time to read. This portion gives some of the objectives and requirements for a successful training program, and shortly I will come back to this for some additional comments.

Section II contains the outlines for the actual training programs for the various segments of the industry. There are six of them, as follows: 1. milk, 2. ice cream, 3. condensed and dry milk, 4. evaporated milk, 5. butter, and 6. cheese. The training program for each of these segments outlines work in these areas: (1) orientation, (2) plant operations, (3) laboratory, (4) engineering, (5) procurement, (6) distribution and sales, and (7) office.

Section III of the manual is a kind of postgraduate course entitled, Management Development. This section is divided into two parts with the first one devoted to the development of skills in personnel management. This part contains information on how to induct the new employee, maintain high morale, handle grievances, reprimand an employee, plan the wise use of the supervisor's time, conduct a safety program, and develop work simplification.

Part 2 of Section III deals with the development of skills in quality control and cost analysis. A large number of study projects have been outlined which, if properly used, should be of inestimable value to a supervisor in obtaining essential information about the operation of the business. It is recommended that these studies be made by a person who has completed his outlined training program. This experience will bring him still closer to the actual operation of the business, especially from a cost-wise point of view. All of the forms suggested for use in these studies have been used in dairy products manufacturing plants and their use has proven effective in improving quality and reducing costs. Such study projects may reveal inefficiencies, the correction of which could save the company considerable money.

An appendix to the manual gives a rather extensive list of reference materials and the names and addresses of trade and professional organizations.

It is recommended that the following procedure be used, to assure complete success in the training program: (1) One person should be assigned the responsibility of serving as the adviser or counsellor to the person during his entire training period. The trainee must have one man to whom he can be directly responsible at all times. Of course, he will work with other supervisors as he progresses from one assignment to another, but he should al-

[1] This manual can be purchased through the office of the Secretary, H. F. Judkins, 32 Ridgeway Circle, White Plains, New York, for $4.00, or from Olsen Publishing Company, Milwaukee, Wisconsin.

ways have this one individual as his chief counsellor. (2) The trainee should be expected to participate in the planned training program in an orderly fashion by following step-wise the training outline. (3) A definite time schedule for the work and training assignments should be established and adhered to. (4) The use of the question approach as recommended in the outline has proved effective and is highly recommended. (5) The trainee should be required to submit periodic reports to his counsellor and be evaluated by a plan outlined in the manual.

It should be emphasized at this time that for each of the training programs, a suggested time schedule be presented. Along with this suggested schedule is a form for recording the adopted schedule, with dates on which the trainee is expected to be assigned to the various training areas. Along with this is space for recording the name of the adviser or counsellor for each of the areas.

Let's return for a moment to the reasons for a training program and some of the requirements for its success. This outline was prepared for the dairy manufacturing graduate. He may or may not have had any commercial experience. Since vocational training is not the objective of a college education, the graduate without a training program will likely find the going quite rough when he assumes his first job in industry. It was the feeling of the committee that one of the immediate objectives of a training program should be to provide an orderly pattern by which the college graduate could bridge the gap between school and industry, and thus find his way without direction. By such an organized procedure, he should be able to utilize much more effectively his more fundamental academic training.

Certainly, an organized training program will prepare the college graduate more quickly for effective supervision of personnel. It would be a serious mistake, as you will agree, to place a college graduate in a supervisory position without practical experience. The training program will assist him to obtain his experience in a formal manner rather than by the unplanned method that exists in plants not having a training program.

May I emphasize that a college graduate should never be employed simply as a permanent member of the working force within the plant. There should be some definite position in mind for him on completion of the training program. Furthermore, management, through interviews, tests, recommendations, etc., should be certain that the graduate is educationally prepared to move into an area of plant operations. His future, therefore, should not be considered in terms of in-plant operations alone.

Of course, this makes it even more necessary that colleges strive to recruit only the top-caliber young men for majors in dairy technology. We should encourage them to take an active part in extracurricular activities, in order that we may have an opportunity to observe their leadership ability, their ability to work with their fellow students, and their general interests in life.

Now, what does it take to make the program successful? Possibly, the most important element is the full and complete endorsement by top management. This means that the top management of the company must initiate the program and see that it is adopted. He must insist that it be carried out according to plan. He must see that adequate supervision of the trainee is supplied, and that one individual serves as the liaison between top management and the trainee. Furthermore, management should encourage and give an opportunity to the trainee to attend and participate in company features that will get him acquainted with the entire organization. This might include participation in educational conferences with company employees, attending special conferences at educational institutions, participation in community programs in which the company takes an active interest, as well as in the social and professional activities of the company.

I stated earlier that the training program was designed with the idea that the college graduate would be the one most likely to make use of it. While this is true, it should be emphasized that the program must be open to all employees of the company, regardless of their educational background.

Management should realize that the program must have considerable flexibility. It must be made to suit the peculiarities of conditions involving either the company or the trainee, or both. The committee feels that this can be done and at the same time stay within the framework of the outline contained in the manual. In any event, a definite schedule should be established and adhered to.

Likewise, management should realize that one of the objectives of the program is to permit the trainee to gain a first-hand view, with appropriate actual experiences, in the entire operation of the company, including office procedures, sales, procurement, etc. This is not intended to train the individual to be an expert in office management, for example, but to give him an understanding of the relationship between the different departments, their responsibilities, problems, etc.

Finally, I would like to emphasize that the program should be on a formal basis, with periodic reports from the trainee to his counsellor. He should be required to have conferences at certain intervals with his counsellor and management. In addition, he should be evaluated on the basis of the form provided in the manual for this purpose. Management and the counselior should keep in mind at all times that the ultimate objective of the training program is the development of supervisory and management personnel for the company.

I can not emphasize too strongly that this manual is only a guide and an outline for a

training program. The questions are obviously too comprehensive to fit all situations in all plants or all departments of a given plant. This should be considered a desirable feature, as it can be adjusted by the simple addition and deletion of questions to make it applicable to any department or to any plant. To criticize the manual because it is too simple, too complex, or because it contains questions that are inapplicable, is to overlook one of the strong features of the plan—namely, to serve as the frame-work of a tailor-made program for an individual plant's needs. This feature should be emphasized to people who plan to use the manual, so they can see its true value and advantage to them.

Past President of the American Dairy Science Association, Dr. A. C. Fay, stressed this point recently when he said, "Any plant manager who wishes to use this Manual as a basis for a training program should certainly add and delete questions, not only to fit his particular operations but to fit the capacity of the trainee. The point is that he has the skeleton of a comprehensive training program at his disposal to assist him in building one that meets his specific needs."

Although the Manual has been on the market less than 6 mo., more than 1,200 copies have been sold already. A few weeks ago I wrote approximately 100 purchasers of the Manual requesting that they express any opinions they may have formed regarding its usefulness, or ways in which they had made use of it already. More than 50 replies have been received to date, and more are being received almost every day. Most of these replies are enthusiastic regarding the influence this outline will have on the company in its personnel training program. The following statements are typical of the comments received:

A Texas plant reports, "If we would devote ourselves to the use of publications such as the Dairy Plant Training Manual, and followed the program as outlined, I am sure we could make a vast improvement over any previous methods we have used."

Another from Texas says, "For several years I have been doing many things the book outlines. I don't think you can beat it, and for a young superintendent it is a great help. I am sure every young man who has started to climb the ladder in a milk plant would appreciate having one of these manuals."

Still from Texas, this plant operator reports, "The Dairy Plant Training Manual is certainly the finest thing of its kind we have ever seen. We plan to use it in our training program after our summer vacations are over."

A New York operator says, "We have no formal training program, but the Manual is used by the production managers for informal training of their supervisors and also by the supervisors for training of first line employees."

A California plant reports, "We have found the Dairy Plant Training Manual very helpful in our training program. It is an excellent guide, not only for training but as an operational reference. We have four trainees in the program."

Another California operator says, "Our opinion is that parts of it are too academic for practical application. An example of this is that the manual suggests time to be scheduled in areas such as office work. A period of two weeks is so inadequate in such work that in four or five years when the person again came to a point in supervision or management where it was necessary to know or use the earlier training, it would have to be repeated. The economy necessary in this industry precludes the use of extended time in any area which is nonproductive."

Of course, my friend is absolutely correct in his appraisal of the economics involved. At the same time, he missed the point completely regarding the purpose of the 2 wk. in the office. This period is not intended to make him an expert in office management, but is intended to acquaint him with the relationship of the office to the other parts of the organization. For example, any person who ever worked in production or quality control has wondered what the purpose was of the forms he was required to fill out daily or perhaps many times daily. What happened to them when they left his department? A brief experience in the office will give him a fuller appreciation of the importance of accuracy in the reports he prepares. Of course, it is anticipated that office procedures will have changed within a period of 5 yr., as was mentioned. If they have not, may heaven help the business, because it is not long for this world!

A report from Tennessee states, "Although the development of management personnel is essential, I feel that too much of the manual's content is devoted to this aspect."

I wonder if he has been looking to the colleges as a source of labor. If so, that is one of the surest ways of ruining the recruitment program of our colleges.

An Alabama plant reports, "The Manual is being used regularly in our plant and we were surprised to get a report from our plant superintendent that he himself had gotten a great deal out of reading the manual, and that he is using it to check standard operating procedures throughout the plant."

A North Carolina operator who is using the manual in his training program says, "I am deeply envious of those who will be fortunate enough to receive their training from the Manual, for in very concise manner you have extracted the salient features of both educational and practical ability and combined it into 119 pages of information. Frankly, this is the only manual I have ever seen that should be bound in gold leaf, for to me it is that important, and I am sure it will be placed to good use in our company."

OUR ASSOCIATION

MAKING THE AMERICAN DAIRY SCIENCE ASSOCIATION MEAN MORE

R. E. HODGSON, President, A.D.S.A.
Animal Husbandry Research Division, USDA, Beltsville, Maryland

It is indeed a pleasure and an honor to serve the membership of the American Dairy Science Association as President during its 56th year of service to the dairy industry. I have the unique opportunity of using the JOURNAL to communicate with you and shall do so from time to time.

In this first communication I desire to report to you some of the work, attitudes, desires, and aspirations of the officers of your Association. I think this can be done most easily by discussing some of the activities and thoughts dealt with at the last meeting of the Executive Board. Let me assure you, however, that this is not the only time the Board and the officers work. Their activities continue in an energetic way throughout the year. Each of the elected and appointed officers is dedicated to giving his best to serving the interests of the Association. This is also true of the more than 150 members who serve on the Association's 31 currently operating permanent and temporary committees and as Association representatives to other organizations. A similar number of you are no less actively serving as officers and committee members of the sections, divisions, and student affiliate chapters of the Association. Thus, about 12 per cent of our membership is enrolled in organized work of your Association. But this is not all. Each of us, whether or not we are a member of one of the committees, works and has a responsibility to work, to advance the objectives of the Association.

The American Dairy Science Association is one of the world's large scientific societies. Its objectives are, through research, education, and technical competence, to advance the welfare of the dairy industry and those engaged in it and to promote human well-being. The membership totals more than 3,200, including the student affiliates. About 250 members live in 37 countries outside the United States.

The Association publishes one of the best scientific journals in the field. Over 4,900 copies are issued each month, with almost 1,400 going to people in foreign countries. It is, no doubt, the greatest source of original dairy technological and educational information to be found any place in the world. In addition, arrangements have been made with the British Commonwealth Agricultural Bureau so that at a nominal cost members receive 12 copies each year of the Dairy Science Abstracts. This publication effectively abstracts the world's dairy science literature. I urge the membership to subscribe to this publication along with the JOURNAL OF DAIRY SCIENCE.

You are a member of a great and influential organization. But this Association must never rest on its laurels. One of the items that your Board gave considerable attention to at its last meeting was how to increase our public relations efforts. What can the Association do to increase the public relations effort so that it can make even greater contributions to the advancement of the dairy industry? This is a question all of us should be concerned with. The Board is thinking of improving public relations not only among the membership at our annual meetings but also public relations affecting other dairy industry associations, groups, individuals, and the public at large.

I shall enumerate a number of actions taken by the Board which we think will advance our efforts along this road. Our excellent awards program was enlarged to include, beginning in 1961, a Distinguished Service Award to be given to individuals who have made outstanding achievements in the dairy industry. The Association this year introduced a new Student Affiliate Section, with its own program at the annual meeting. The Board is encouraging the re-establishment of the general session at the annual meeting, the development of the dairy products extension session, with the possibilities of making this a separate section, and further development of the educational session at the annual meeting. The Board is looking into the possibility of developing a dairy economics section. The Board is endeavoring to enlist the interest of people engaged in work relating to dairy economics and management, with the view of having more papers in these areas presented at our annual meetings and in the JOURNAL OF DAIRY SCIENCE. Each of the Section Chairmen is being invited to sit in and discuss his program and activities before the Board meetings.

Another activity the Board is engaged in is to explore the desirability of initiating a Sustaining Membership program for the Association. In the international field, the Board authorized a committee to explore ways and means of achieving better international relations and understanding with dairy scientists throughout the world. Direct communication is being made with each member residing outside of the United States and Canada.

The Board feels that these recent actions,

together with the excellent relations that the Association already enjoys with many other dairy industry associations and groups and other technical societies, will improve our public relations efforts and service. In developing these programs the Board has had the full cooperation and assistance of the Policy Committee and many other committees of the Association.

In the final analysis, however, it is the responsibility of each member of our Association to demonstrate and exemplify what the Association stands for and to render such service as he can to advance the objectives of the Association.

As your President, I ask you to write me if you have questions about the Association and to write to the JOURNAL Editor or to any officer of the Association expressing your opinions on Association affairs. The JOURNAL is an excellent medium for the members to exchange communications and should be used for this purpose.

CALL FOR PAPERS FOR THE 1961 ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION[1]

G. W. TRIMBERGER
Chairman, Program Committee, A.D.S.A.
Department of Animal Husbandry, Cornell University, Ithaca, New York

The 56th Annual Meeting of the American Dairy Science Association will be held June 12-14 at the University of Wisconsin, Madison. Members who wish to present papers must submit titles and abstracts not later than March 1. This deadline must be met to permit publication of titles with the complete program in the May issue and abstracts in the June issue of the JOURNAL. Last year all but four abstracts arrived on time. If a large number of papers are suggested for presentation, those that arrive late may be assigned to be read by title or rejected entirely.

Abstracts must contain no more than 200 words by actual count. Abstracts longer than 200 words cannot be accepted and will be returned to the author(s) by the Committee. An original (on bond paper) and three copies of each abstract must be typed double-spaced. The original and one copy should be mailed to the Chairman, another copy to the Vice-Chairman, and the fourth to the Secretary of the Section before which the paper is to be presented. Eventually, the original copy will be sent to the Editor for publication in the JOURNAL.

In the preparation of abstracts, the style and abbreviations of the JOURNAL OF DAIRY SCIENCE must be used. Please refer to abstracts in the June, 1960, issue of the JOURNAL for guidance. Only initials of authors should be used, except in unusual cases where it may be necessary to use the complete name. The title of the abstract should indicate clearly the nature of the research. It should not be repeated again in the text. The abstract should include, insofar as possible, the design and major results of the investigation. Only completed research should be reported. Brief, essential statistics will make the data more meaningful.

All members of the Association are entitled to present papers. Participation by members of the industry and by senior members of the Association is particularly encouraged. Attention is called to the Dairy Manufacturing Extension Section program, and members are urged to participate. This is a subsection of the Manufacturing Section for 1961. The competitive presentation of papers by graduate students, initiated in the Production Section in 1959 and in the Manufacturing Section in 1960, will be continued in both sections.

Papers submitted for the Annual Meeting should be confined to research that has not been reported previously. Abstracts of research articles accepted for publication by a scientific journal before the annual meeting are not acceptable, because this constitutes a duplication. The Program Committee favors the general policy that an individual present only one paper and that his name appear as author on no more than two. The Program Committee, together with the Association membership, wishes to stimulate vigorous, sound research and realizes that the above restrictions may penalize some members engaged in full-time research. Therefore, the Committee has liberalized this policy to the extent that a member can exceed these limits, but only if he or his department rates the abstracts in order of preference for oral presentation. If the total number of papers submitted by the membership is too great to include in the program, the Committee will assign some papers to be read by title only. In this event, consideration will be given to quality of abstract and research reported therein, number of abstracts per author and department, and importance of the research reported.

The Program Committee encourages contin-

[1] The procedures for preparing abstracts will apply also to abstracts submitted for the Eastern, Southern and Western Divisional Meetings of the American Dairy Science Association. Editor-in-Chief.

ued improvement in quality of papers and in oral presentation. Careful design of experiments and proper interpretation of results are necessary prerequisites. Strict compliance with the instructions for preparation of abstracts will simplify the task of the Program Committee, and will improve the program. Careful editing of abstracts before submission is essential. In the past too many abstracts have been lacking in this respect.

All abstracts must be submitted on regular 8½ by 11-in. paper. The following form, with no capitals for the title, is correct and is in accordance with the form used in 1960:

> Utilization of carbohydrates posterior to the rumen-reticulum of the bovine. J. T. Huber and N. L. Jacobson, Iowa State University.

If the author lists an address for an experiment station other than the University, or a USDA research branch, or a commercial company, then the complete address should be provided, as presented in the following example from the 1960 program:

> A study of dye reduction methods as platform tests for the detection of antibiotics. Burdet Heinemann, Producers Creamery Co., Springfield, Missouri.

Preparation of mimeographed copies of pertinent data is desirable. At least 250 copies should be available for distribution. This can be supplemented by slides for projection on screens, provided the author can adhere to the assigned time of 12 to 14 min. for presentation of each paper.

Names and addresses of officers of sections to whom titles and abstracts should be sent are:

EXTENSION SECTION

Chairman: M. E. Senger, Department of Animal Industry, North Carolina State College, Raleigh.
Vice-Chairman: W. R. Van Sant, Department of Dairy Science, University of Arizona, Tucson.
Secretary: C. D. McGrew, Department of Dairy Science, Ohio State University, Columbus, 10.

PRODUCTION SECTION

Chairman: S. D. Musgrave, Department of Dairying, Oklahoma State University, Stillwater.
Vice-Chairman: J. C. Thompson, Ralston Purina Co., St. Louis, Missouri.
Secretary: L. H. Schultz, Department of Dairy Husbandry, University of Wisconsin, Madison.

MANUFACTURING SECTION

Chairman: Burdet Heinemann, Producers Creamery Co., Springfield, Missouri.
Vice-Chairman: M. L. Speck, Department of Animal Industry, North Carolina State College, Raleigh.
Secretary: D. M. Graham, Pet Milk Co., Research and Development Center, Greenville, Illinois.

(The Dairy Manufacturing Extension Section is a temporary subsection in the Manufacturing Section. R. B. Redfern, Dairy Extension, North Carolina State College, Raleigh, is chairman and W. S. Arbuckle, Department of Dairy Husbandry, University of Maryland, College Park, is secretary. All abstracts for papers in the Extension Section should be submitted through the regular channels of the Manufacturing Section, but should be identified for presentation at Extension Section meeting and a courtesy copy be sent to R. B. Redfern.)

Summer meeting of Eastern Division, American Dairy Science Association, August 8-9, 1960, University of New Hampshire, Durham.

Officers: Prof. K. S. Morrow, Host, University of New Hampshire. Dr. Frank Potter, Vice-Chairman, East. Div. A.D.S.A., University of Mass., Amherst; Dr. Eldon Johnson, President, University of New Hampshire; Dr. H. A. Keener, Secy.-Treas. East. Division, A.D.S.A., University of New Hampshire; Dr. B. R. Poulton, President East. Div. A.D.S.A., University of Maine, Orono; Dr. H. F. Judkins, Secy.-Treas. A.D.S.A., White Plains, New York; Dr. A. C. Fay, Past President, 1959-60, of A.D.S.A., Miami, Florida.

ABSTRACTS OF PAPERS PRESENTED AT EASTERN DIVISION MEETING

UNIVERSITY OF NEW HAMPSHIRE, DURHAM, AUGUST 8 AND 9, 1960

Antibiotic residues in milk. C. J. COSGROVE AND W. M. ETGEN, University of Rhode Island, Kingston.

A study was conducted to determine if antibiotic residues were present in the untreated quarters of cows following treatment of one quarter and the duration of antibiotic residues following intramuscular injection. Thirty-three cows were used in the study of intramammary treatment, 25 receiving intramuscular penicillin injections. Milk samples, taken at each subsequent milking, were analyzed daily by disc assay methods. These tests continued until the samples exhibited a negative reaction. Of the 25 cows given intramuscular penicillin injections in amounts varying from 3,000,000 to 6,000,000 units per cow, four did not show any antibiotic residue in the milk, 12 had residues for one milking, five for two milkings, two for four milkings, one for three milkings, and one for six milkings. Only four of the 33 animals, given intramammary infusions of antibiotics, had residues in the milk from the untreated quarters. One had residues for one milking, two for two milkings, and one for three milkings. The milk from the treated quarter of the cows in this group contained antibiotic residues from four to 15 milkings.

Observations on the incidence of penicillin transfer from treated to untreated quarters of cows' udders following infusion of penicillin for treatment of mastitis. D. A. EVANS AND D. N. STERN, University of Massachusetts, Amherst.

Studies were undertaken to determine the possible incidence of penicillin transfer from treated to untreated quarters of cows' udders after direct udder infusion of penicillin for treatment of mastitis. Eight indivdual cows in different stages of lactation were used. The animals were treated with maximum prescribed separate dosages of aqueous base and oil base procaine penicillin G over a three-day period. Samples of milk from the individual quarters of the treated cows were collected at each milking following infusion and analyzed for penicillin, using the Standard Disc assay procedure.

Penicillin, when carried in either an aqueous or an oil suspension, was transferred to untreated quarters in similar amounts. Penicillin concentration in untreated quarter or quarters was greatest in low-producing animals and least in higher-producing animals. Relative rates of excretion of penicillin from the untreated quarters was greatest in high-producing animals and least in low-producing animals.

Comparison of the sensitivity of three disc assay procedures for detection of specific antibiotics added to milk. M. J. WILLIAMS, E. WEINGARTEN, AND D. A. EVANS, University of Massachusetts, Amherst.

Studies were undertaken to determine some of the limitations and applications of three disc assay procedures used for detection of antibiotics in milk. These included the Rapid 3-hr. test developed by Arret and Kirshbaum, the procedure described in Standard Methods for the Examination of Dairy Products, tenth edition, and a modification of the latter employing prolonged incubation at lower temperature.

Results indicated that the Standard Methods procedure and the Rapid 3-hr. procedure gave comparable results with respect to the concentration of antibiotic detectable, and can be used with equal satisfaction. The modified Standard Methods procedure, however, was not as efficient in detecting lower concentrations of antibiotics as the two other methods. It was observed that the prolonged incubation period probably resulted in the overgrowth of narrow inhibition zones by resistant organisms.

Comparative influence of consuming silage versus breathing the odor, on feed flavor in milk. A. Bradfield and H. M. Allen, University of Vermont, Burlington.

The objective of this study was to ascertain how much of the feed flavor (silage) in milk was caused by the cow eating the silage and how much was caused by breathing the odor of silage.

Cows were fed 20 lb. of good quality silage at various times before milking. They consumed this in 30 min. The next day some of the same silage was placed in a feed bag which had holes in the side and a fine screen over the silage. The bag was hung over the cow's head for 30 min. Thus, the cow was able to breathe the odor but could not eat the silage. This was done at the same time before milking. Samples were tested organoleptically immediately after the next milking.

When the cows ate the silage, the flavor score was about one point lower than when they only breathed the odor. The lowest score in both cases occurred when the cows ate the silage or breathed the odor 2½ hr. before milking.

It was evident that although eating silage gave the most severe off-flavor, breathing the odor was almost as effective.

The flavor of milk from individual cows. F. E. Potter and D. J. Hankinson, University of Massachusetts, Amherst.

From March to July 15, 1960, 2,955 samples of milk from individual cows were judged for flavor. These samples were obtained monthly from 26 herds of the Ayrshire, Guernsey, Holstein, Jersey, and Brown Swiss breeds. They were taken by DHIA testers and were usually from 24 to 48 hr. old when tested. The flavor distribution for all samples was 10.1% no criticism, 46.5% feed, 23.1% oxidized, 4.6% rancid, 8.7% salty, 2.2% cowy, and 4.8% miscellaneous flavors. The milk samples from the Jersey and Guernsey breeds had about twice as many no-criticism samples as the other breeds. Feed flavors were about equally distributed for all breeds, but the salty flavor was noticeably higher for the Ayrshire and Jersey breeds. The rancid flavor progressively decreased from 12.8% in March to 1.9% in July. Samples with a feed flavor varied from 41.9% in March to 54.1% in May.

Observations on several aspects of milk quality after addition of solutions of dairy sanitation chemicals. H. V. Atherton, University of Vermont, Burlington.

Residuals of sanitation chemicals have concerned the dairy industry for over 30 yr. New methods of milk production and new sanitation chemicals have created renewed interest in the effect of these residues on milk quality.

Samples of farm bulk cooled milk were adulterated to contain 0, 1, 5, and 10% of added cleaner and sanitizer solution at manufacturer's recommended strength. Bacterial analyses at plate incubation temperatures of 20 and 32° C. for 48 ± 3 hr., coliform counts (VRBA) at 35° C. for 18 hr., and organoleptic determinations of flavor were made following adulteration with 19 products and holding for zero, two, and four days of raw storage at 5° C. None of the products significantly affected the bacteria counts of milk. Household detergents and certain dairy cleaners seriously affected milk flavor. Dairy sanitizer residues seldom reduced milk flavor noticeably at concentrations anticipated with accidental contamination. Low level (1%) contamination occasionally produced serious flavor defects not found in samples containing a higher degree of adulteration.

Pipeline milker studies. Some factors affecting the development of rancidity. G. W. Gander and R. G. Jensen, Storrs Agricultural Experiment Station, Storrs, Connecticut.

The complete milkings from one or more selected cows were passed through a full-size experimental pipeline milker to determine the effect of certain factors on the development of rancidity. Acid degree values were measured by the silica-gel method on samples taken from the mixed milk and past the pump in the pipeline milker and stored for 24 hr. at 4° C. The variables and the resulting average acid degree values were: (a) level of milk intake into pipeline; control—1.24, floor level —1.89, line level—1.96; (b) air leaks; control —1.62, 2.05; (c) vacuum level without air leaks; control—0.77, 7 in.—1.07, 12 in.—1.21; with air leaks; control—1.17, 2.03, 2.42; (d) addition of antifoaming agent; none—1.54, added—1.66.

The freezing point of milk from individual cows. F. E. POTTER AND SHUNROKURO ARIMA, University of Massachusetts, Amherst.

The freezing point on 2,863 samples of milk from individual cows was determined during the period of March to July, 1960. The samples were taken by DHIA testers from 26 herds and were from 24 to 48 hr. old at the time of analysis by a Fiske Cryoscope. Freezing points varied from −.515 to −.577° C. Seventy-five and seven tenths per cent of the samples exhibited freezing points between −.540 and −.560° C. The number of samples tested and the average freezing point by breed are as follows: Ayrshire, 577 samples, −0.550° C.; Guernsey, 664 samples, −0.554° C.; Holsteins, 665 samples, −0.549° C.; Jersey, 606 samples, −0.553° C.; Brown Swiss, 351 samples, −0.552° C. One per cent of all samples had freezing points above −0.530° C.

Effect of carbon dioxide removal upon the freezing point of vacuum-treated milk. J. M. MOORE AND A. C. SMITH, Storrs Agricultural Experiment Station, Storrs, Connecticut.

Vacuum treatment of fluid milk by nonsteam injection flavor removal equipment resulted in an elevation of the freezing point at low levels and a depression at high levels of flash-cooling when homogenized milk was processed at 172°F. in various adaptations of a vacuum unit. The loss of carbon dioxide at the reduced pressures employed for the vaporization of the volatile flavor components was found to be the primary factor responsible for the elevation of the freezing point at low levels of flash-cooling.

Vacuum removal of carbon dioxide from milk was accomplished under laboratory conditions without concentration of the milk. Under these conditions, the elevation of the freezing point value was represented by the equation, logarithm of $Y = -0.293 + 0.010\ X$, where Y denotes the freezing point elevation and X the carbon dioxide volume per cent removed from the milk.

Comparison of methods for determining solids-not-fat and protein content of milk. A. R. CORWIN, F. R. ALLAIRE, JR., AND S. N. GAUNT, University of Massachusetts, Amherst.

The small Watson Lactometer, Golding Plastic Spheres, and Mojonnier methods were compared for the determination of solids-not-fat (SNF) per cent on individual cow samples of milk. A correlation of .94 was obtained between the Golding and Lactometer methods on 134 samples, .87 between Golding and Mojonnier, and .82 between Lactometer and Mojonnier, on 108 samples. The lowest correlation was found in Jerseys and the highest in Holsteins. The means for the Watson Lactometer, Golding, and Mojonnier methods for SNF were not significantly different. Protein determinations by Kjeldahl and Formol Titration (Oregon method) were made on the same samples. The pooled correlation on four breeds was .88. Effects of age and temperature on the Golding and Watson Lactometer methods for determining SNF were investigated. Milk samples were stored at 40 and 55° F. for zero, one, three, and six days. No significant differences for the effects of age or temperature were found. Standard errors of estimate for the first and third day determinations were ± .17% for the Golding Plastic Spheres and ± .23% for the Watson Lactometer.

Effect of pelleted hays on the growth and development of dairy heifers. F. S. HAZLETT, R. W. HEMKEN, AND R. F. DAVIS, University of Maryland, College Park.

Feed consumption and growth rate of dairy heifers fed long hay and ground pelleted hay were compared. Mature timothy hay and third-cutting alfalfa harvested at one-tenth bloom and at full bloom were studied. A total of 48 heifers were used in two experiments of 103 and 113 days. Heifers fed pelleted hay consumed 32% more hay and had a daily weight gain and increase in height at withers of 45 and 27% more, respectively, than those fed long hay. The presence or absence of straw bedding had no effect on these factors with heifers fed timothy hay. Stage of maturity of the alfalfa hay or grinding through a ¼-in. screen vs. a ⅛-in. screen also had no effect. Increased rate and efficiency of gain observed for heifers fed pelleted hays appeared to be associated with increased intake.

Digestibility of fiber in alfalfa and timothy hay. J. G. ARCHIBALD, D. F. OWEN, JR., H. FENNER, AND H. D. BARNES, University of Massachusetts, Amherst.

Four Holstein cows were divided into two groups of two each and fed alfalfa hay and timothy hay by the double-reversal feeding system for four 6-wk. periods during the winter of 1959-60. Hay only was fed at the rate of 2.5 lb. per 100 lb. of body weight.

Four digestion trials of ten-day duration were conducted, using the total feces collection procedure. Results are available from eight individual trials with each kind of hay.

Major differences in composition were the higher fiber, cellulose, and pentosan content, and the lower lignin content of the timothy hay. The principal point of interest is the highly significant difference ($P<.001$) in fiber digestibility in favor of the timothy. Dry matter and cellulose of the timothy were also significantly more digestible. The reason for these rather striking results, confirming as they do the value of good grass hays for milk production in contrast with grass-legume hays at this station over the past 4 yr. is due to a relatively

high degree of lignification in the alfalfa hay which was a second cutting. The timothy hay was an early first cutting.

A study of the effects of date of harvest on the nutritive value of timothy forage. T. N. MELIN, B. R. POULTON, AND M. J. ANDERSON, University of Maine, Orono.

First-cutting timothy hay was harvested at 11 stages of maturity beginning on May 27 and at seven-day intervals until August 5. The analysis of the forages showed, on a dry matter basis, a crude protein content of 28.81% for the May 27 cutting, which decreased almost uniformly to 8.10% for the July 22 cutting and then remained approximately the same for the last two cuttings, as did all nutrient values determined. The dry matter digestibility of the forage was 81.63% for the May 27 cutting and decreased at a near constant rate to 51.98% for July 22. A regression equation was calculated for dry matter digestibility and was found to be $Y = 84.91 - 0.481\ X$ where X is equal to the number of days after May 17 that the forage was harvested.

Protein digestibility ranged from 84.9% for the May 27 cutting to 51.6% for the July 29 cutting. The digestible energy of the forages in Cal/g, and the apparent digestibility of the energy, showed a uniform drop from 3.70 and 79.3%, respectively, for the May 27 cutting, to 2.30 and 49.3% for the July 22 cutting.

Effect of stage of maturity at first cutting on yield and nutritive value of forages. S. L. SPAHR, E. M. KEELER, J. B. WASHKO, AND J. W. BRATZLER, The Pennsylvania State University, University Park.

Alfalfa-timothy-clover was cut for hay in 1959 on May 25 (Hay 1), June 9 (Hay 2), and June 24 (Hay 3). Orchard grass was cut on May 25 (Hay 4) and June 9 (Hay 5). Aftermath yields were obtained from the field of mixed forage. The first-cutting hays were used in a digestion trial with sheep, and in a lactation trial which employed 14 Holstein cows.

Seasonal yields of TDN were 2,040, 3,140, and 3,530 lb/A when the mixed forage was cut initially on May 25, June 9, and June 24, respectively. Digestible energy was 69.2, 62.6, 58.3, and 63.8 for Hays 1 through 4, respectively. In terms of 4% FCM per cow daily production Hays 1 through 5, respectively, was 40.4, 37.1, 32.8, 34.3, and 31.4 lb. Average daily hay intakes (90% D.M.) per 100 lb. of body weight were 2.92, 2.63, 2.24, 2.29, and 1.68 lb. Orchard grass was about 2 wk. more mature than the mixed forage cut on the same date. Milk production, body weight changes, per cent digestible energy, and maximum intake were more closely associated with stage of maturity than with date of harvest.

Variations in the glucosamine content of cervical mucus during the estrous cycle. W. M. ETGEN AND C. DESJARDINS, University of Rhode Island, Kingston.

This study was conducted to determine (a) if glucosamine was present in detectable quantities in the cervical mucus of dairy cattle, and (b) whether variations occurred in the amount present during the estrous cycle. Daily cervical mucus samples were taken and analyzed for glucosamine content from four normal-cycling, nonpregnant dairy cows for a period of 25 days. Mucus samples were also taken at 8-hr. intervals and analyzed for glucosamine content from each of ten cows. This began approximately three days before heat and continued for three days after heat. Results indicated that glucosamine was present during the entire estrous cycle. The amount was relatively low and constant except for a brief time following the heat period. The glucosamine content began to rise about the time of cessation of heat, reached the highest concentration approximately 12 hr. later, and returned to a relatively low level within 30 hr. after the cessation of heat. From these results, it appears that a high concentration of glucosamine might be associated with ovulation in dairy cattle.

Glucosamine content of cervical mucus throughout the estrous cycle in dairy cattle. R. H. FOOTE, W. C. WAGNER, J. V. BARTFAY, AND L. C. GRAY, Cornell University, Ithaca, New York.

An increase in glucosamine in cervical mucus has been reported to be an indication of ovulation time in humans. Using a modification of the Elson and Morgan and the Kurzrok procedures, glucosamine content was determined on 45 samples from cows at various stages of the estrous cycle and of samples from ten cows throughout their estrous cycles. Mucus was obtained from the external os of the cervix by aspiration with Frank's cervical mucus pipette, with 2- by 2-in. gauze tampons, and with a modified bacteriological loop about 7-8 mm. in diameter. It was difficult to obtain sufficient mucus for analysis during diestrus. Slight contamination of the mucus with blood interfered with the test. The concentration of glucosamine ranged from 0 to 2.0 mg. per gram of mucus. Glucosamine concentration tended to be higher during diestrus than at estrus, with considerable individual variation. Therefore, the level of glucosamine in cervical mucus is not a reliable indicator of estrus in dairy cattle.

Effect of dyes, colored tubes, and catalase on the survival of bovine spermatozoa agitated and exposed to light at 5° C. R. H. FOOTE

AND L. C. GRAY, Cornell University, Ithaca, New York.

The motility of spermatozoa is decreased by agitation and exposure to oxygen and light, all factors involved in routine handling of bull semen. One to 200 μg. of lyophilized catalase per milliliter of CUE extender improved survival of spermatozoa stored at 5° C. The sperm from 15 bulls stored in the dark for eight days at 5° C. with 0, 10, and 100 μg. of catalase per milliliter averaged 51, 53, and 57% motile. The same samples exposed continuously to a light intensity of 200 footcandles from fluorescent bulbs averaged 28, 37, and 39% motile. Similar samples stored in the dark versus the light, but mixed and opened twice daily, averaged 41, 45, and 47 in the dark and 0, 3, and 20% motile in the light. Subsequent studies showed that coating the storage tubes with transparent green dye, as well as the addition of catalase, partially protected spermatozoa exposed to light. As expected, inclusion of green dye in the extender had no protective action.

Correlation between certain laboratory studies and fertility of bovine spermatozoa. B. W. PICKETT, W. A. MACDONALD, D. G. GOSSLEE, AND W. A. COWAN, University of Connecticut, Storrs.

Two laboratory studies were conducted in conjunction with a field trial in an attempt to develop a method of evaluating the relative fertility of frozen semen prior to field use. In the laboratory studies, two storage temperatures were employed, −76 and −196° C., while the fertility data were collected employing only dry ice storage. In the first study, paired samples from the two storage temperatures after 2 hr., 90, and 180 days storage were thawed in ice water and examined for per cent progressive motility (P.M.) immediately and at 1, 3, 6, and 12 hr. post-thaw. Simple correlations between motility and fertility at −76° C. storage indicated per cent P.M. estimations at 1, 3, and 6 hr. at zero time were an indication of fertility ($P<0.05$). In the second study, ampules of frozen semen from −76 and −196° C. storage were exposed to ambient temperatures for 0, 1, 2, 3, 4, or 5 min., returned to their respective refrigerant, and allowed to equilibrate 3 hr. before motility estimation. This procedure was repeated after 15, 30, 60, and 120 days storage. Significant simple correlations were found ($P<0.05$) between fertility and per cent P.M. cells after 3 hr. storage at −76° C. and exposure for 1, 2, 3, or 4 min.

Effect of extra udder stimulation on milk production. W. S. GAUNYA AND C. E. BUTLER, University of Connecticut, Storrs.

It has been suggested that the extra milk ejection responses associated with three- and four-time milking could possibly account for the increased production observed in three- and four-time milking.

An experiment was conducted to determine the effect of extra udder stimuli on milk production. A 9-wk. double-reversal design with 16 cows was employed. The rate of concentrate feeding was individually established at the onset of the experiment and maintained at this level throughout the trial. Grass silage and alfalfa hay were fed ad lib. The experimental treatment consisted of an udder wash with warm water, applied half-way between each milking interval. Fore milking was not practiced, to eliminate the possibility of milk leakage.

One of the cows was withdrawn because of illness. Average production for the 15 cows during the stimulation periods was 34.1 lb. of milk or 35.9 lb. of 4% FCM. During the control periods, productions were 33.6 and 35.9 lb., respectively. Extra udder stimulation had no effect on milk production.

A comparison of the mastitogenic effect of two types of milking machine liners, one molded and one stretched. J. M. MURPHY, University of Pennsylvania, Philadelphia.

An experimental method has been designed to use small numbers of cows (eight) examined over a period short enough (12 wk.) that new infection was not likely to occur. The absence of inflammation in uninfected cows, and the degree of inflammatory activity in already infected cows, was measured by the leucocyte content of milk from each cow at every milking. The cows were divided into two groups handled the same in all ways except that one was milked with a common brand of molded teat-cup liner and the other with a common brand of two-piece stretched (extruded) liner. The type of liner was reversed at the end of each 3-wk. period, resulting in a two-group, double-reversal treatment. There was no significant difference in the mastitogenic effect of the two types of liners, as shown by clinical symptoms of mastitis. Analysis of leucocyte counts, however, revealed that two infected animals responded more favorably to the stretched liner.

Relative errors in some body measurements of dairy cattle. R. E. MATHER, P. F. RANDEL, AND A. A. RIMM, New Jersey Agricultural Experiment Station, Sussex.

Five sets of data on 99 heifers from 16 to 28 mo. of age and 52 milking cows were analyzed to determine the relative errors of body weight and five body measurements. The average error variance (based on duplicate measurements) as percentages of total variance (animal plus error) for the different measures were: body weight, 0.7%; hip width,

0.5%; chest depth, 1.3%; heart girth, 2.2%; wither height, 3.7%; and paunch girth, 5.8%. For body length (withers to hips) average error variance was 8.2% for two heifer groups. Rank of the percentage error variances seemed to justify recording body weight to the nearest 2 lb., hip width and wither height to the nearest 0.1 cm. or 1/16 in., heart girth and wither height to the nearest 0.2 cm. or 1/8 in., and paunch girth to the nearest 0.5 cm. or 1/4 in. In addition to small errors, hip width and chest depth would also be desirable in many cases due to negligible effects of body condition, fill, and position, and are recommended for greater use in research.

Mineral problems encountered in farm herds. R. S. ADAMS, The Pennsylvania State University, University Park.

Refusal to eat forage apparently resulted in symptoms of stiffness, founder, and constipation in a dairy herd in a quarry area. Unpalatable legume hay contained below normal amounts of Ca and P, and unusually high levels of Si. Problems which appeared on a farm after 3 yr. of an all-grass program using 100-300 lb. N/A as well as P and K, will be discussed. Low blood serum levels of Ca, inorganic P, K, Mg, and Na were found. No typical symptoms of nitrate poisoning were observed. Levels of KNO_3 equivalent in forage dry matter ranged from 0.066 to 0.528%. No problems were noted with Holsteins fed an average of 40 lb. per head daily of green-chopped corn forage containing 0.891-4.993% KNO_3 equivalent (D.M. basis) over a period of 5 wk. Numerous abortions were reported in a two-county area where drouth-stricken, immature corn was ensiled. Corn silage which caused typical nitrate poisoning symptoms contained only 0.354% KNO_3 equivalent. In another herd slobber forage was encountered with first cutting bird's-foot trefoil pasture, as well as second-cutting clover silage and hay.

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